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## CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 30 March 2004 with an application for Letters Patent number 532057 made by FAC8CELL PTY LIMITED.

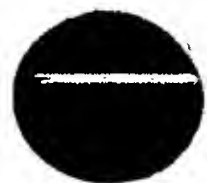
I further certify that pursuant to a claim under Section 88 of the Patents Act 1953, a direction was given that the application proceed in the name of OLGA GARKAVENKO;ROBERT BARTLETT ELLIOTT;ALFRED VASCONCELLOS;DWAINE EMERICH;CHRIS THANOS.

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**PROVISIONAL SPECIFICATION**

**“METHODS FOR CULTURING CELLS AND USES THEREOF”**

We, FAC8CELL PTY LIMITED, a company duly incorporated under the laws of Australia of 160 Greenhill Road, Parkside, South Australia 5063, Australia, do hereby declare this invention to be described in the following statement:

## TECHNICAL FIELD

The invention relates to the use of secretory cells for the treatment of diseases associated with a deficiency in a secreted factor. More particularly but not exclusively it relates to methods of isolating and culturing secretory cells, enhancing the survival of secretory cells, and use of secretory cells, for the treatment of such diseases, and in particular, to the isolation, characterization, culturing, and use of liver cells.

Using the methods described herein, isolated secretory cells retain for extended periods the capability of mediating cell-specific functions. Therefore, isolated secretory cells may have a wide range of applications, including, but not limited to, their use to augment, replace and/or reconstitute a functionally deficient secretory organ by, for example, implantation, or isolation of secreted factors.

## BACKGROUND OF THE INVENTION

Many diseases, deficiencies and conditions can be treated by supplying to the patient one or more biologically active factors produced and/or secreted by living cells, or removing from or reducing in the patient deleterious factors which are metabolized by living cells. In many cases, these factors can restore or compensate for the impairment or loss of organ or tissue function. Examples of diseases or conditions whose etiologies include loss of secretory organ or tissue function include, but are not limited to, diabetes, wherein the production of insulin by pancreatic islets of Langerhans is impaired or lost, hypoparathyroidism, wherein the loss of production of parathyroid hormone causes serum calcium levels to drop resulting in severe muscular tetany, hypothyroidism and cretin dwarfism, both due to thyroid hormone deficiency, hypophysial dwarfism due to pituitary growth hormone deficiency, Lesch-Nyhan Syndrome due to hypoxanthine-guanine phosphoribosyltransferase deficiency, fulminant hepatic failure due to the hepatotropic factor deficiency, hemophilia A, wherein a deficiency in the production and/or secretion of the blood clotting factor VIII can lead to severe bleeding episodes, Parkinsonism, wherein dopamine production is diminished; and

anemia, which is characterized by the loss of production of red blood cells secondary to a deficiency in erythropoietin.

In some diseases, conditions or deficiencies whose etiologies lie in the loss or diminishment of a single biologically active factor, replacement or replenishment of said factor may be therapeutically effective. For example, administration of insulin to diabetics can significantly improve the control of serum glucose and delay the onset of complications associated with elevated serum glucose.

However, the impairment or loss of organ or tissue function may result in the loss of multiple metabolic functions. For example, it has been reported that in fulminant hepatic failure, liver tissue is rendered incapable of removing toxins, excreting the products of cell metabolism, and secreting essential factors, such as albumin and Factor VIII (Bontempo, et al., Blood, 69, pp. 1721-1724 (1987)).

In many diseases or conditions, the affected organ or tissue is one which normally functions in a manner responsive to the physiological state, by, for example, response to fluctuations in the levels of specific metabolites and/or physiologically important substances, thereby maintaining homeostasis. For example, pancreatic islet  $\beta$ -cells normally modulate production of insulin in response to fluctuations in serum glucose. Traditional therapeutic approaches to the treatment of such diseases cannot compensate for the responsiveness of the normal tissue to these fluctuations. For example, an accepted treatment for diabetes includes daily injections of insulin. This regimen cannot compensate for the rapid, transient fluctuations in serum glucose levels produced by, for example, strenuous exercise, or ingestion of food. It has been suggested that failure to provide such attuned responsiveness to the physiological state may lead to complications of the disease state.

Many other diseases are, likewise, characterized by a deficiency in a biologically active factor that cannot easily be supplemented by injections or longer-term, controlled release therapies. Still other diseases, while not characterized by substance deficiencies, can be treated with biologically active factors normally made and secreted by cells. Thus, trophic and growth factors may



be used to prevent neurodegenerative conditions, such as Huntington's and Alzheimer's diseases, and adrenal chromaffin cells which secrete catecholamines and enkephalins, may be used to treat pain.

Accordingly, many investigators have attempted to reconstitute organ or tissue function by transplanting whole organs organ tissue which provide secreted products or affect metabolic functions. For example, liver transplantation is the established therapy for end-stage liver disease, as described by Starzl, et al. N. Eng. J. Med. 321:1014-1022 (1989). In another example, patients with hemophilia A have undergone liver transplantation as a result of liver failure resulting from hepatitis acquired from the blood derived factor VIII. In these instances, there has been a complete cure of the hemophilia. Transplantation can provide dramatic benefits, but is limited in its application by the scarcity of organs available for transplantation. For example, it has been reported that approximately 30,000 people die each year in the United States of liver disease (American Liver Foundation, Vital Statistics of the United States, 1988; Vol. 2(A)), and 23% of those listed for transplantation in 1991 died while waiting for an organ (Annual report of the U.S. scientific registry for organ transplantation and the organ procurement and transplant network, 1990. Richmond, Va., UNOS, and Bethesda, Md., the Division of organ transplantation, Health Resources and Services Administration, PE59, 19).

In general, the patient must undergo immunosuppression or immunomodulation in order to avert immunological rejection of the transplant, which results in loss of transplant function and eventual necrosis of the transplanted organ or tissue. However, immunosuppressive or immunomodulatory therapy generally impairs the patient's overall immunological defenses, which may increase susceptibility to the risks of a variety of serious complications, including nephrotoxicity, neurotoxicity, hypertension, increased susceptibility to infection and osteoporosis. Moreover, this approach is not always effective in altering the course and incidence of rejection episodes. Typically, the transplant must remain functional for a long period of time, even for the remainder of the patient's lifetime. It is both undesirable and expensive to maintain a patient in an immunosuppressed or immunomodulated state for a substantial period of time.

Transplanted cells provide the potential for treating various diseases because the cells can provide factors to replace or supplement natural factors which, due to their insufficiency or absence, cause disease. Cell implantation therapy has an advantage over traditional factor-supplementation therapy regimens because the transplanted cells are responsive to the physiologic state, by, for example, response to fluctuations in the levels of specific metabolites and/or physiologically important substances in the recipient. The release of therapeutic factors from the transplanted cells may be properly regulated provided the transplanted cells have the necessary receptors and ability to respond to endogenous regulators.

Patients having disease as a result of the loss or deficiency of secreted factors, such as proteins, enzymes, hormones, neurotransmitters, growth factors or other physiological substances are considered to be among those who would achieve significant benefits from transplant therapy. For example, implantation of pancreatic islet cells could provide insulin as needed to a diabetic.

Because cells which are implanted may be foreign to the host it is necessary to prevent the host immune system from attacking and thereby causing the death of the implanted cells. Various immunosuppressive or immunomodulatory methods to protect the implanted cells by attenuating the host immune response are possible. However, these methods suffer the disadvantages associated with immunosuppression or immunomodulation therapies used in conjunction with organ transplantation. There is the further disadvantage of potentially producing adverse effects on the transplanted cells, for example, impairing their engraftment and/or function and/or reducing their secretory responses.

A desirable alternative to such attenuation procedures involves the isolation of the implanted cells from effectors of the recipient's immune response by, for example, implantation within a physical barrier which will allow diffusion of nutrients, waste materials, and secreted products, but block the cellular and molecular effectors of immunological rejection. A variety of methods which isolate implanted cells from the immune system have been explored. These include the use of extravascular diffusion chambers, intravascular diffusion chambers, intravascular ultrafiltration chambers, and implantation of microencapsulated or

macroencapsulated cells. Such methods have the potential to obviate the need to subject the patient to immunosuppression or immunomodulation therapy.

However, none of these approaches have been satisfactory for providing long-term transplant function. A method of delivering biologically appropriate quantities of needed factors or providing other needed metabolic functions for an extended period of time is still unavailable and would be very advantageous to those in need of long-term treatment.

It would therefore be advantageous to have a method for enhancing the survival of and/or maintenance of function of liver cells in tissue culture for an extended period.

It would furthermore or alternatively be advantageous to have a method for enhancing the survival and/or maintenance of secretory function of liver cells capable of secreting one or more factors in tissue culture for an extended period.

It is an object of the invention to provide methods of preparing and culturing liver cells and/or enhancing the survival of and/or maintenance of liver cells in tissue culture for an extended period of time, or to provide those in need of said methods with useful choice.

## **STATEMENTS OF THE INVENTION**

According to a first aspect of the invention there is provided a method for the long term culturing of liver cells, said method comprising or including preparing a cell preparation comprising or including liver cells and culturing said cell preparation in media comprising autologous serum.

In one embodiment, the liver cells are secretory liver cells.

Preferably, the liver cells are selected from one or more of hepatocytes, non-parenchymal liver cells, sinusoid cells, liver vein cells, gall bladder cells, gall bladder epithelial cells, gall bladder endothelial cells, liver endothelial cells, and

In one embodiment, the culturing is in the presence of companion cells capable of providing a growth and/or trophic and/or mitogenic function to said liver cells.

Preferably, the said companion cells are selected from one or more of sertoli cells, fibroblasts, ovarian cells analogous to sertoli cells.



Preferably the companion cells are selected from one or more of Sertoli cells, fibroblasts, growth-arrested fibroblasts, ovarian cells analogous to Sertoli cells, and genetically modified cells.

In one embodiment, the secretory liver cells are hepatocytes, preferably neonatal hepatocytes.

Preferably the hepatocytes are isolated from cell cultures such as those available from Cell Dynamics LLC (Smyrna, Georgia, USA).

In an alternative embodiment, the secretory liver cells are gall bladder cells, preferably gall bladder endothelial and/or epithelial cells.

In a further alternative embodiment, the secretory liver cells are selected from one or more of non-parenchymal liver cells, sinusoid cells, liver vein cells, liver endothelial cells, and

Preferably one or more of the factors is a blood clotting factor.

Preferably the blood clotting factor is Factor VIII.

More preferably the blood clotting factors are both Factor 8 and 9.

Preferably one or more of the factors is a growth and/or differentiation factor.

Preferably the one or more secreted factor is a hormone.

Preferably the growth and/or differentiation factor is selected from growth hormone and analogues thereof, insulin like growth factor and analogues thereof, hepatocyte growth factor and analogues thereof, or fibroblast growth factor and analogues thereof.

Preferably the hormone is a corticosteroid.

According to a second aspect of the invention there is provided **a method for maintaining the phenotype of an isolated, purified or recombinant secretory liver cell**, said method comprising or including preparing a secretory liver cell preparation and culturing said preparation in media comprising autologous serum.

In one embodiment, said cell preparation comprises secretory liver cells and companion cells capable of providing a growth and/or trophic and/or mitogenic function to said secretory liver cells.

According to a third aspect of the invention there is provided **one or more secreted factors** prepared as described herein with or without reference to the examples and/or figures.

#### **DESCRIPTION OF THE FIGURES**

**Figure 1** depicts a graph of factor VIII and albumin production by a hepatocyte preparation as described in Example 1 herein.

**Figure 2** depicts a graph of albumin production by hepatocyte preparations as described in Example 4 herein, wherein ARF+FBS indicates hepatocytes grown in media supplemented with 10% FBS in the presence of mitomycin arrested fibroblasts, ARF+PS indicates hepatocytes grown in media supplemented with 10% PS in the presence of mitomycin arrested fibroblasts, ARF NO S indicates hepatocytes grown in serum-free media in the presence of mitomycin arrested fibroblasts, COLL+PS indicates hepatocytes grown in collagen coated flasks in media supplemented with 10% PS, C+PS+F indicates hepatocytes grown in collagen coated flasks in media supplemented with 5% PS and 5% fibroblast-conditioned growth media, C+PS+S indicates hepatocytes grown in collagen coated flasks in media supplemented with 5% PS and 5% Sertoli-conditioned growth media.

**Figure 3** presents a photomicrograph of ICG positive cells in culture, as described in Example 4 herein.

**Figure 4** presents a photomicrograph of ICG staining of adult mouse hepatocytes in culture, as described in Example 6 herein.

**Figure 5** presents a photomicrograph of ICG staining of adult mouse hepatocytes in culture, as described in Example 6 herein.

**Figure 6** presents a photomicrograph of ICG staining of adult mouse hepatocytes in culture at x20 magnification, as described in Example 6 herein.

**Figure 7** presents a photomicrograph of alginate capsules with single mouse hepatocytes at x4 magnification, as described in Example 6 herein.

**Figure 8** presents a photomicrograph of alginate capsules with single mouse hepatocytes at x4 magnification, as described in Example 6 herein.

**Figure 9** presents a photomicrograph of alginate capsules with a spheroid of mouse hepatocytes at x4 magnification, as described in Example 6 herein.

**Figure 10** presents a photomicrograph of alginate capsules with mouse hepatocytes at x4 magnification with dark ground condenser, as described in Example 6 herein.

**Figure 11** presents a photomicrograph of alginate capsules with mouse hepatocytes at x10 magnification with dark ground condenser, as described in Example 6 herein.

**Figure 12** presents a photomicrograph of alginate capsules with mouse hepatocytes at x4 magnification with dark ground condenser, as described in Example 6 herein.

**Figure 13** depicts a graph presenting albumin production by fibroblasts in growth medium supplemented with porcine serum (F+PS), hepatocytes in growth media supplemented with porcine serum (Hep+PS), hepatocytes in media supplemented with foetal bovine serum (Hep+FBS), as described in Example 7 herein.

**Figure 14** depicts a graph presenting cell counts of hepatocytes isolated by our standard procedure and by the cold ischemia method, as described in Example 8 herein.

**Figure 15** depicts a graph presenting albumin production by hepatocytes isolated by our standard procedure and by the cold ischemia method, as described in Example 8 herein.

**Figure 16** depicts a graph presenting factor VIII production by hepatocytes isolated by our standard procedure and by the cold ischemia method, and by gall bladder cells, as described in Example 8 herein.

#### **DETAILED DESCRIPTION:**

The methods and preparations of the instant invention are useful for long-term culturing of liver cells, including secretory liver cells. Such long term culturing

of liver cells has application in various methods including the provision of a wide range of biologically active factors to an individual in need of them, and/or to provide needed metabolic functions to an individual, such as the removal of harmful substances. Biologically active factors provided by the methods and preparations of the invention include a wide variety of molecules normally secreted by the liver. For example, Factor VIII (FVIII) can be delivered to a Type A hemophiliac, or  $\alpha$ 1-antitrypsin can be delivered to a patient with  $\alpha$ 1-antitrypsin deficiency.

The methods and preparation described herein can also be used to restore or augment vital liver-mediated metabolic functions, such as the removal of toxins or harmful metabolites (e.g., cholesterol) from the bloodstream by liver cells such as for example, hepatocytes or non-parenchymal liver cells, or gall bladder epithelial or endothelial cells. The methods of the invention make possible the implantation of liver cells despite a shortage of donor tissue or organs. Through use of the methods of this invention, homeostasis of particular substances and/or metabolic function can be restored and maintained for extended periods of time.

Loss of or reduction in liver function is responsible for a great number of diseases, conditions and deficiencies. For example, inborn errors of metabolism relating to the liver individually are rare but collectively are common. The biological basis of the majority of inborn errors of metabolism relating to the liver is single gene defects, which result in abnormalities in the synthesis or catabolism of proteins, carbohydrates, or fats. Most inborn errors of metabolism relating to the liver are due to a defect in a biological factor, such as an enzyme or protein, which leads to a block in a metabolic pathway. Pathophysiological effects most commonly result from toxic accumulations of substrates before the block, accumulation of intermediates from alternative metabolic pathways, and/or defects in energy production and utilization caused by a deficiency of products beyond the block.

For example, hemophilia A results from an inherited deficiency of clotting factor VIII, normally produced by the liver. When less than 1% of normal factor VIII activity exists in the blood, severe bleeding episodes in response to minimal trauma occur.



Hemophilia affects about 1/10,000 live births around the world. About 1/3rd of these cases are in the severe category.

The established treatment is replacement by injection of the missing FVIII. Isolated FVIII was originally derived in semipurified form from blood, and was thereby subject to the problems associated with blood-derived products, such as being a potential source of infectious agents, such as HIV, or Hepatitis B and C. Blood-derived FVIII has in part been replaced by recombinant factor VIII.

Ideally, FVIII is given prophylactically, but therapy is very expensive (about \$100,000/ year). Furthermore, neutralizing antibodies may be generated in the patient, inhibiting the activity of the injected factor.

Occasionally, patients with hemophilia A have undergone liver transplantation as a result of liver failure resulting from hepatitis acquired from the blood derived FVIII. In these instances, there has been a complete cure of the Hemophilia.

As with many diseases of other organs, liver transplantation is often a preferred therapy in diseases associated with errors of liver metabolism. For example, liver transplantation is the established therapy for end-stage liver disease. However, as with most other transplant therapies, liver transplantation is limited by the scarcity of suitable donor organs.

The transplantation of hepatocytes has been proposed as an alternative to whole organ transplantation for liver disease (Asonuma, et al. J. Ped. Surg., 27:298-301 (1992)). The authors report that single metabolic deficiencies may be cured with replacement of 12% of liver mass, suggesting a single liver could be utilized for several patients, or partial resection of a living donor's liver could provide the necessary liver mass to treat another person.

The ability of transplanted cells to manufacture and secrete substances of therapeutic value, or provide needed metabolic functions, and so potentially provide an alternative to whole organ transplantation, has led to the development of implantable devices for maintaining cells within an individual in need of treatment.

In order to replace or augment liver function utilizing liver cell transplantation, regardless of the means of cell delivery, it is critical to ensure the



survival and growth of the transplanted cells. Previous studies on hepatocyte transplantation have reported that performing a portal caval shunt (PCS) in conjunction with hepatocyte transplantation improves hepatocyte engraftment (Uyama, et al., Transplantation 55:932-935 (1993)). However, patients in need of liver function replacement, such as hemophiliacs or patients in liver failure, are already in a compromised situation, and the burden of a PCS may not be feasible for this population.

The invention disclosed herein provides methods for enhancing the survival and/or maintenance of function of liver cells for long term culture *in vitro*. The invention disclosed herein in respect of secretory liver cells, including for example, human hepatocytes, and companion cells relates in a preferred embodiment to the preparation and culturing of a co-culture of Sertoli cells and/or fibroblasts with human hepatocytes.

Prior art methods involving the use of Sertoli cells and other cells, such as islets, have generally involved processing and isolation of each separately and putting together at the time of the transplant.

We have found that preparation and co-culturing of liver cells and companion cells allows the liver cells to grow in the presence of growth factors produced by the companion cells *in vitro*. For example, we have found that hepatocytes survive and function better if they are protected by the layer of companion cells, for example, Sertoli cells or fibroblasts.

We have further found that preparation and culturing of liver cells *in vitro* in autologous serum enhances the survival of and/or maintenance of liver cell function in long term cultures. For example, we have found that pig hepatocytes survive and function better if they are grown in porcine serum.

We have additionally found that preparation and culturing of liver cells *in vitro* in media supplemented with factors produced by companion cells enhances the survival of and/or maintenance of liver cell function in long term cultures. For example, we have found that pig hepatocytes survive and function better if they are grown in media comprising fibroblast-conditioned growth media.

Ideally both the liver cells and companion cells are derived from the same donor species.

By "companion cells" as used herein we mean cells capable of enhancing survival and/or viability and/or biological function of the secretory cells with which they are cultured and/or aggregated. For example, the companion cells may provide growth and/or trophic and/or mitogenic factors to said secretory cells

By "secretory cells" we mean liver cells, including hepatocytes or non-parenchymal cells, and/or gall bladder cells, and/or genetically modified cells, capable of performing metabolic functions normally performed by the liver and/or expressing and secreting a biologically active molecule. The biologically active molecule may be selected from but not limited to one or more of the following; blood clotting factors (for example, FVIII), growth and/or differentiation factors (for example, growth hormone and analogues thereof, insulin-like growth factor and analogues thereof, hepatocyte growth factor and analogues thereof, fibroblast growth factor and analogues thereof), and hormones (for example, corticosteroids).

By "liver cells" we mean cells from the liver or associated organs, including the gall bladder, bile duct and hepatic vessels, including, for example hepatocytes, non-parenchymal liver cells, gall bladder epithelial cells, gall bladder endothelial cells, and liver vessel endothelial and epithelial cells.

By "secretory liver cells" we mean cells from the liver or associated organs, including the gall bladder, bile duct and hepatic vessels, capable of secreting one or more factors normally secreted by the liver or associated organs.

By "autologous serum" we mean serum for cell culture derived from the same species as that from which the cells were derived. In examples of the present invention where secretory cells and companion cells from different species are co-cultured, an autologous serum is one derived from the same species as that from which the secretory cells were derived.

By "factor" we mean a biologically active molecule produced by a cell.

By "long term" we mean a period of more than a week, typically extended to 2 -6 weeks or more.

Co-culturing of liver cells with companion cells, for example, Sertoli cells isolated from the testes of donor subjects, has been investigated as a means of achieving an enhancement or stimulation of longevity and/or growth and/or the mitotic rate of liver cells such that they release higher amounts of factor and/or survive longer and/or proliferate *in vitro*.

Sertoli cells are known to play a critical role in various physiological activities such as the synthesis of certain growth factors, for example, insulin-like growth factors 1 and 2 (IGF-1, IGF-2, respectively) and epidermal growth factor (EGF), immunomodulation, possibly as a result of increased secretion of transforming growth factor-beta 1 (TGF- $\beta$ 1), and an anti-apoptotic (cell death inhibitory) function.

Our recent studies in experimental animal models have shown that the presence of companion cells, such as Sertoli cells, improves the *in vitro* functional competence of islet cells, and that transplantation of islet-sertoli cell aggregates prolongs islet cell survival. The precise mechanism by which Sertoli cells protect islet cell grafts against immune rejection is not precisely known, but we believe, without wishing to be bound, it to be related to the production of growth and differentiation factors by Sertoli cells.

Our invention relates in part to the long term co-culturing of companion cells, such as Sertoli cells, with liver cells, and in particular secretory liver cells, such as hepatocytes, *in vitro* such that the companion cells can act as "nursing" cell systems for the secretory cells, providing enhancement of their functional performance and longevity.

This approach is complementary to, and synergistic with, other approaches for providing functional longevity for transplanted secretory cells.

The maintenance of liver cell function and enhancement of liver cell survival achieved through the co-culturing of liver cells and companion cells is demonstrated herein. For instance, Example 2 herein discloses the effects of co-culturing hepatocytes with growth arrested and non-arrested fibroblasts on the maintenance of a secretory cell phenotype.

The invention further relates in part to culturing liver cells, and in particular secretory liver cells, such as hepatocytes, in the presence of autologous serum and/or companion cell-derived factors. The Applicants believe, without wishing to be bound, that autologous serum is able, through the provision of growth and/or trophic and/or mitogenic factors, an enhancement of liver cell functional performance and/or a maintenance of liver cell phenotype and/or longevity *in vitro*. Similarly, the Applicants believe that companion cell-derived factors are able to provide an enhancement of liver cell functional performance and/or a maintenance of liver cell phenotype and/or longevity *in vitro*.

The effects of culturing liver cells in media supplemented with autologous serum on the maintenance of liver cell function and enhancement of liver cell survival is demonstrated herein. For instance, Example 2 herein discloses the effects of culturing pig hepatocytes in media supplemented with porcine serum on the maintenance of a Factor VIII secretory cell phenotype.

Similarly, the effects of culturing liver cells in media supplemented with companion cell-derived factors on the maintenance of liver cell function and enhancement of liver cell survival is demonstrated herein. For instance, Example 2 herein discloses the effects of culturing pig hepatocytes in media supplemented with fibroblast-derived growth media on the maintenance of a Factor VIII-secretory cell phenotype.

The following Examples are provided to illustrate but not to limit the invention in any manner.

## **EXAMPLE 1**

### *1.1 Isolation of hepatocytes and optimisation of culture conditions*

Hepatocytes were isolated from neonatal porcine liver using our standard procedure as follows. Following surgical removal, the donor livers are transferred to a clean room facility for further processing in a cold plastic container in 50ml tubes containing cold Hank's Balanced Salt Solution (HBSS) with 0.2% human serum albumin (HAS) added. The liver cells are isolated by digestion of the minced liver via a major modification of the standard (Ricordi's) collagenase digestion procedure. Using aseptic technique, the liver is trimmed of excess fat, blood vessels



and connective tissue, minced, and digested with Liberase® (0.2 mg/ml) in a shaking water bath (120 rpm) for 10 minutes. The digestion step is repeated twice. The digestion is performed using lignocaine mixed with the Liberase® solution to avoid cell damage during digestion. Following the digestion process, the cells are passed through a sterile 400µm mesh into a sterile beaker. Following the isolation, liver cells are placed into tissue culture in various media as described herein.

To optimise the culture conditions for survival of liver cells and the maintenance of a secretory cell phenotype, different media supplemented with various additives were assessed.

Hepatocytes were grown in different liquid media on a number of different surfaces and on surfaces coated with different matrices. Optimum growth was found with the medium DMEM/F 12 (1: 1 by volume) plus, for example, the additives cyproxin, nicotinamide (10mmol/L), insulin, glucagons (7ng/mL), hydrocortisone (7.5µg/mL) and autologous serum (10% by volume). The best surface matrix for hepatocyte growth was found to be collagen.

### *1.2 Maintenance of secretory cell phenotype in culture*

To determine whether liver cells in culture maintain liver cell function, for example, the ability to produce or secrete liver cell secreted factors,

Hepatocytes, isolated from neonatal porcine liver according to our standard protocol, were cultured in the preferred liquid medium for up to 5 weeks on a matrix of collagen as described above. The production of Factor VIII and albumin was then determined as follows.

250,000 hepatocytes per flask were cultured in medium supplemented with additives. Supernatant from the cultured cells was removed and discarded, and cultures were washed twice with PBS. 5 ml serum free media was then added to the culture flasks. A 1 ml aliquot of media was immediately removed from the flask and used for a base line measurement. Cells were then incubated at 37°C for 4 hours. After incubation, supernatant was collected, and filtered to remove cellular debris. Albumin present in the filtered supernatant was measured using the pig albumin ELISA Core Kit (Komabiotech) following the manufacturer's protocol.



Factor VIII present in the filtered supernatant was measured using the Dade-Behring clotting system.

Production of Factor VIII was observed, with cells producing considerable quantities after 2 weeks in culture and maintaining the output of Factor VIII for 5 weeks (see Figure 1) at which time the experiment was terminated.

The output of 250,000 hepatocytes over a 4-hour period gave a Factor VIII value approximately 8% of normal blood levels. Since the half life of Factor VIII in human blood is 36 hours, this rate of production is very substantial. It should also be noted that the production of albumin by these hepatocyte preparations correlates well with Factor VIII production. Albumin is a typical liver product, and production of albumin indicates hepatocytes are healthy.

These results indicate that neonatal hepatocytes are able to maintain a secretory liver cell phenotype during prolonged cell culturing.

## EXAMPLE 2

The effects of manipulation of the secretory cell environment by, for example, modulation of cell-extracellular matrix interactions, different cell-cell interactions, or addition of soluble stimuli, on secretory cell growth and function *in vitro* was assessed as follows.

### 2.1 Cell-extracellular matrix interaction

The effect of interactions between the secretory cells and an extracellular matrix on cell viability and maintenance of cell-specific function *in vitro* was assessed using collagen as exemplary extracellular matrix.

Hepatocytes were isolated following our standard procedure. Aliquots of hepatocytes were put into a collagen-coated flask and a non-coated flask. DMEM growth media (GM) supplemented with 0.5U /mL, glucagons (7ng/ mL), hydrocortisone (7.5ug/mL), and 10% (by volume) porcine serum (PS) was used. The effect of autologous serum supplementation on cell viability and function in the presence of a secretory cell-extracellular matrix interaction was assessed using as a control hepatocytes grown in a collagen coated glass in growth media lacking porcine serum.

Photomicrographs of the cell cultures were taken after 15 and 18 days in culture.

At both 15 and 18 days of culturing, hepatocytes grown in the presence of a collagen extracellular matrix formed a confluent monolayer irrespective of the presence or absence autologous serum in the growth medium. In contrast, hepatocytes grown in the absence of a collagen extracellular matrix formed multiple foci. The functional significance of the different *in vitro* morphologies observed was evaluated with a test on hepatocyte function. Growth media was collected from each flask every fourth day for an analysis of albumin production to check the function of hepatocytes during culture. Results for albumin production are presented in Example 4 herein.

## 2.2 Cell-cell interaction

Human fibroblasts were used as companion cells to study the role of cell-cell interaction in fibroblast-hepatocyte co-cultures. Growth-arrested fibroblasts and non-arrested fibroblasts were co-cultured with hepatocytes under the following experimental conditions: 700 000 non-arrested fibroblasts : 250 000 hepatocytes in GM supplemented with 10% fetal bovine serum (FBS) or 10% PS; a confluent monolayer of fibroblasts arrested with mitomycin C : 250 000 hepatocytes in GM supplemented with 10% FBS or 10% PS.

Cell morphology was assessed by photomicrography after 10, 16, 30, 37 days in co-culture. For each cell preparation, a flask in which the cells were cultured in serum-free GM was used as a control. The effect of a 3-dimensional support structure on the cell-cell interactions was also investigated, using hepatocytes grown on nylon mesh coated with mitomycin arrested human fibroblasts. Briefly, nylon mesh was successfully coated with human fibroblasts. The fibroblasts were then arrested with mitomycin. Hepatocytes were placed in the flask with the mesh. After 5 days the mesh was washed with fresh medium and put into a new flask.

Non-arrested fibroblasts quickly overgrew hepatocytes. Cells formed a confluent monolayer even in GM without sera. The control flask with hepatocytes

grown in GM without sera was empty after 7 days in culture (data not shown), indicating these conditions could not support hepatocyte survival.

Hepatocytes grown with arrested fibroblasts in GM supplemented with 10% PS presented better morphology compared to cells in GM supplemented with 10% FBS. Cell viability as assessed by morphology was optimal after culturing for 2-3 weeks.

### *2.3 Optimisation of growth media.*

The effect of supplementation of growth media with various sera on hepatocyte growth and function was assessed. Hepatocytes (250 000 cells per flask) in collagen coated flasks were grown under the following experimental conditions: GM supplemented with 5% PS and 5% Sertoli-conditioned growth media prepared as described below. GM with 5% PS and 5% pig skin fibroblast-conditioned growth media prepared as described below, and 10% PS.

Sertoli-conditioned growth media was prepared as follows. Sertoli cells were cultured for at least 24 hours before growth media was collected and filtered through an 8 micron filter to remove cells. The filtered media was then diluted in a 1:1 ratio with DMEM before use.

Pig skin fibroblasts were isolated as follows. Pig skin was soaked in DMEM plus cyproxin, and fungizone, for 20 minutes, then cut with a scalpel into small pieces. Pieces of tissue were then placed in a standard culture flask with DMEM media supplemented with 10% PS. After one week of culture, pieces of tissue were removed, the remaining cells adhering to the flask were washed, and fresh growth medium was added.

Fibroblast-conditioned growth media was prepared as described for Sertoli-conditioned growth media above, with the substitution of fibroblasts for Sertoli cells.

Supplementation of growth media with 10% PS, or 5% PS and 5% pig skin fibroblast-conditioned growth media, yielded better hepatocyte viability than supplementation of growth media with 5% PS and 5% Sertoli-conditioned growth media. Cell viability as assessed by morphology was optimal after culturing for two to three weeks, as in Example 2.2 above.

Analysis of the morphology of the cells in culture showed that for hepatocyte viability and growth *in vitro*, supplementation of growth media with autologous (porcine) serum was preferable to supplementation with foetal bovine serum. Furthermore, supplementation of growth media with either 10% PS, or 5% PS and 5% fibroblast-conditioned growth media was preferable to supplementation with 5% porcine serum and 5% Sertoli-conditioned media.

### EXAMPLE 3

#### *Cryopreservation of hepatocytes*

Hepatocytes were isolated according to our standard procedure described above, and additionally according to our modified procedure, in which digestion with Liberase® during isolation is performed in media supplemented with PS. The isolated hepatocytes were pooled, and then frozen following the standard procedure described under the following three different conditions: in 10%DMSO in FBS; 10% FBS and 10% DMSO in GM; and 10% DMSO in PS. Cells were stored in liquid nitrogen. After one week, and three weeks storage, cells were defrosted and viability and recovery were determined. Results are summarised in Table 1.

Table 1. Hepatocyte cryopreservation

	Viability	Cells cultured for 3 days		Cells cultured for 30 days	
		Viability	Recovery	Viability	Recovery
PS/DMSO Modified isolation	96%	83%	72%	80%	60%
GM/DMSO	96%	60%	43%	76%	30%
FBS/DMSO modified isolation	96%	81%	64%	ND	ND
PS/DMSO conventional isolation	57%	61%	45%	0%	0%

Viability is the percentage of living cells. Recovery is the percentage of living cells after thawing.

Cells maintained good viability and recovery after storage in liquid nitrogen for one to two weeks. The best viability and recovery of cryopreserved hepatocytes was observed with hepatocytes isolated according to our modified procedure and frozen in 10% DMSO in PS.

#### **EXAMPLE 4**

##### *Maintenance of secretory cell phenotype in culture*

##### *4.1 Albumin secretion*

Albumin is the major plasma protein secreted by hepatocytes. In conventional cultures, the rate of secretion of albumin drops rapidly. Hepatocyte albumin secretion was used herein as a test for the maintenance of normal hepatocyte function, and thus the maintenance of a secretory cell phenotype, in culture.

Media harvested from hepatocytes grown under the various conditions described in Example 2 above, (and reproduced below), were analysed for albumin content, as an indication of hepatocyte function.

- 1- Hepatocytes on mitomycin arrested fibroblasts in growth media supplemented with 10% FBS, see Example 2.2;
- 2- Hepatocytes on mitomycin arrested fibroblasts in growth media supplemented with 10%PS, see Example 2.2;
- 3- Hepatocytes on mitomycin arrested fibroblasts in growth media without serum, see Example 2.2;
- 4- Hepatocytes on collagen coat in growth media supplemented with 10% PS, see Example 2.1;
- 5- Hepatocytes on collagen coat in growth media supplemented with 5% PS and 5% fibroblast conditioned growth media, see Example 2.3;
- 6- Hepatocytes on collagen coat in growth media supplemented with 5% PS and 5% Sertoli conditioned cells growth media, see Example 2.3 as follows.

Aliquots of supernatant were taken at day 5, 7, 10, 13, 32 and 54 of culture, as follows. First, supernatant from the cultured cells was removed and discarded, and cultures were washed with PBS. 5 ml of growth media without serum was then added. A 1 ml aliquot of this growth media was immediately taken from the flask,



and used for a baseline measurement for albumin production. Cells were then incubated at 37°C for four hours. After incubation, supernatant was collected and filtered to remove cellular debris. Albumin present in the filtered supernatant was measured using pig albumin ELISA Core Kit (Komabiotech) according to the manufacturer's protocol. Growth media prepared in accordance with the original culture conditions was then added to the flasks for continued culturing.

As show in Figure 2, the highest albumin production was observed in cultures supplemented with porcine serum. Hepatocytes grown in media supplemented with PS on arrested fibroblasts yielded a maximum albumin release of 19.5 µg/ml for 4 hours at day 10 in culture (see Figure 2). Hepatocytes grown on collagen matrix in GM supplemented with PS showed a maximum albumin release at day 32 in culture of 38.3 µg/ml for 4 hours.

Maximum albumin production for hepatocytes grown in GM supplemented with PS and with fibroblast-conditioned growth media was 3.76 µg/ml at day 32 of culture. Maximum albumin production of hepatocytes grown in GM supplemented with PS and sertoli-conditioned growth media was 4.56 µg/ml at day 13 in culture.

These results demonstrate that hepatocyte secretory function, as assessed by albumin production was best maintained when the hepatocytes were grown in collagen coated flasks in growth media supplemented with 10% PS. A secretory cell-extracellular matrix interaction and the presence of autologous serum are important for the maintenance of a secretory cell phenotype in long term tissue culture.

#### 4.2 Indocyanine Green Uptake

Indocyanine green (ICG) is a non-toxic organic anion that is used in clinical tests to evaluate liver function, as it is eliminated exclusively by hepatocytes *in vivo*. ICG uptake has been used to identify differentiated hepatocytes from stem cells in culture (Yamada et al., 2002). In the present study, cellular uptake of ICG was used to identify hepatocytes in culture, in a screening method to identify the best culture conditions for the long term maintenance of hepatocyte function.

The ICG solution (DMEM with 10% PS) was added to the cell culture flask and incubated at 37°C for 15 min. After the flask was rinsed three times with PBS, the cellular uptake of ICG was examined by microscopy. After the examination, the flask was refilled with fresh growth media.

Microscopic examination of isolated neonatal hepatocytes showed that approximately 50% of cells were ICG positive (see Figure 3).

The maintenance of hepatocyte function in long term tissue culture can be readily assessed by IGC uptake. Furthermore, administration of ICG to hepatocytes in tissue culture is a useful methodology to manipulate hepatocyte function and control hepatocyte differentiation *in vitro*.

#### EXAMPLE 5

The importance of the procedures used to isolate secretory cells from tissue to the maintenance of secretory cell function was assessed using Factor VIII secretion and albumin production as markers for liver secretory cell function. The ability of non-parenchymal (NPC) cells from the liver, and epithelial and endothelial cells from gall bladder and liver vessels, to exhibit and maintain a secretory cell phenotype in culture was also assessed. Methods to assay Factor VIII activity and to identify cell populations in culture were also developed.

One neonatal (one week old) piglet, and one approximately 6 month old pig were used in the following experiments. The supernatant of cultured cells was harvested as indicated, and albumin release and FVIII functional tests (ELISA, and coagulation test as described herein, respectively) were performed. ICG uptake tests were also performed as indicated.

##### 5.1 Secretory function of different liver cell types

Hepatocytes were isolated from one neonatal pig liver following our standard procedure described herein, with two rounds of digestion with Liberase® (0.2mg/ml) for 10 min. After isolation, cells were counted and plated in 25 cm<sup>2</sup> flasks, with  $4.5 \times 10^6$  cells/flask. Cells were counted after 5 days in culture, viability was checked by trypan blue exclusion as described above. Albumin, and FVIII release was assessed at day 5 in culture.

The production of Factor VIII was measured using a Factor VIII coagulation assay (Coatest VIII:C/4 from Chromogenix) according to the manufacturer's protocol. Percent values for the rate of Factor VIII coagulation are relative to the rate of Factor VIII coagulation at normal blood levels of Factor VIII.

$37 \times 10^6$  hepatocytes were isolated from one neonatal pig liver as described above. Viability of the cells immediately after isolation was 98%. After 5 days in culture, cell survival rate averaged 60%. Albumin production after 5 days in culture was 4.45 ug/ml/4h. The maximal rate of FVIII coagulation was 0.2%. Staining with indocyanin green was used to identify the percentage of hepatocytes in cell culture. 43% of cells were ICG positive in normal liver preparation after 4 weeks in culture.

NPC were isolated following the procedure by Gerlach et al. (2001), with modifications. Briefly, the liver was cut into small pieces, washed three times to remove erythrocytes. Tissue was then digested with Liberase® (0.2mg/ml) for 30 min. Digestion was stopped with 10% porcine serum. Hepatocytes were sedimented at 50 g for 5 min. Non-parenchymal cells were sedimented at 600 g for 10 min, and then washed three times in PBS. Cells were counted, and their viability was checked by trypan blue exclusion as described above. Cells were plated at 10,000 cells/flask. At day 7 in culture, the cell count and viability check was repeated. Supernatant was collected for albumin ELISA, and FVIII functional tests.

10,000 NPC were isolated from the same neonatal pig liver. Viability of cells immediately after isolation was 100%. Maximal rate of FVIII coagulation was 0.2% at day 5 in culture.

Epithelial and endothelial cells were isolated from pig gall bladder and liver vessels as follows. Briefly, gall bladder was thoroughly washed with sterile DMEM to remove bile, cut into pieces, and digested with Liberase® (0.2 mg/ml) for 30 min. Cells were then washed with DMEM three times. Cell count and viability tests were performed immediately after isolation, and again at day 7, day 16, and day 28 in culture. Cells were plated in 25cm<sup>2</sup> flasks at  $15 \times 10^6$  cells/flask. Albumin and FVIII release functional tests were performed at day 7 in culture.

$31 \times 10^6$  cells were isolated from pig gall bladder and liver vessels. Viability immediately after isolation was 100%. After 16 days in culture, cell survival rate

was 120%. Maximal albumin production was 2.27 ug/ml/4h, and the maximal rate of FVIII coagulation was 3.7%.

### 5.2 Effect of isolation method

*Cold Ischemia.* The effect of cold ischemia during cell isolation on secretory cell function were assessed as follows. Liver was cut into small pieces, and put in a large volume of cold DMEM for 24 hours storage at 4°C. Hepatocytes and NPC were then isolated following the standard procedure as described herein.

251 x 10<sup>6</sup> hepatocytes were isolated from one pig liver using the cold ischemia method described above. Viability immediately after isolation was 21%, with 52 x 10<sup>6</sup> cells surviving isolation. After 4 weeks in culture survival rate was 18%. Maximal FVIII coagulation rate was 1% after 4 weeks in culture.

444 x 10<sup>6</sup> NPC were isolated from the same liver. Viability immediately after isolation was 6.3%. After isolation using the cold ischemia procedure, 95% of cells in culture were positive for ICG.

*Warm Ischemia.* The effect of warm ischemia during cell isolation on secretory cell function was assessed as follows. Liver was cut into small pieces, and put into a large volume of DMEM and stored at room temperature for 6 hours and for 12 hours. These cells were cultured for approximately 7 weeks.

After seven weeks in culture hepatocytes isolated using the warm ischemia method as described above were releasing FVIII into the supernatant. Maximal rate of FVIII coagulation for cells prepared according to the standard procedure was 1.6%. For cells prepared using 6 hours of warm ischemia, maximal rate of FVIII coagulation was 2.7%, and for cells prepared using 12 hours of warm ischemia, maximal rate of FVIII coagulation was 1.2%. One should take into consideration that the flask with hepatocytes isolated using 12 hours of warm ischemia was almost empty with a very small cell count.

*Abattoir Liver.* Three methods of cell isolation were assessed, using tissue from a single organ collected from an abattoir. The liver was collected and immediately put in cold Hank's solution supplemented with antibiotics and fungizone. Within one hour of collection, the tissue had been processed following the standard procedure, described herein. One aliquot of cells was put on a Percoll



gradient, another one on Lymphoprep system, while the third aliquot was treated as per routine procedure.

Isolation of liver cells from the abattoir liver gave a very poor yield and quality of cells. The best isolation was achieved with the Lymphoprep system. Notwithstanding this, due to the very poor yield of viable cells, functional tests were not conducted.

### *5.3 Identification of cell populations in cultures*

An immunoperoxidase method to distinguish different liver cell populations in culture was developed. Adult pig and neonatal pig livers were stained with hepatocyte specific antigen and von Willebrand factor to distinguish endothelial cells and hepatocytes. The following markers were used: hepatocyte antigen and cytokeratin for mature hepatocytes, von Willebrand factor for endothelial cells, vimentin for cells of mesenchymal origin, and Factor VIII to identify cells that are the main producer of the factor.

Freshly isolated liver cells or tissues were formalin fixed, paraffin embedded, and sectioned at 2  $\mu\text{m}$ . Unstained slides were deparaffinized in xylene and hydrated in graded alcohols. Slides were treated with 0.5%  $\text{H}_2\text{O}_2$  for 5 min to block endogenous peroxidase activity. Sections were stained with primary antibody using the DAKO EnVision System according to the manufacturer's protocol. Sections were incubated for 30 min with primary antibody, followed by 30 min incubation with peroxidase-labelled polymer, and 5 min incubation with substrate-chromogen. Slides were counterstained with hematoxylin.

### *Discussion*

The Applicant's standard isolation technique utilising low Liberase® concentration results in good cell yield and viability.

Excellent results in respect of cell yield and secretory cell function were achieved using the cold ischemia method developed by the Applicants and described herein. The number of isolated cells was higher using the cold ischemia method than after isolation using the normal method, with  $52 \times 10^6$  viable cells compared to  $37 \times 10^6$  viable cells using the standard preparation methodology.



After normal preparation, 43% of cells were positive for ICG, a marker for functional hepatocytes. In comparison, 95% of cells were positive for ICG using the cold ischemia method. The FVIII coagulation test showed that cells isolated using the cold ischemia method produced more FVIII, (1%) compared to cells isolated using the standard method (0.2-0.5%).

Excellent results in regard to hepatocyte secretory function were achieved using the warm ischemia isolation method described herein. FVIII coagulation showed that hepatocytes cultured for approximately 7 weeks following isolation using 6 hours of warm ischemia yielded 2.7% of normal FVIII function, compared to 1.6% for hepatocytes isolated using the standard protocol. Hepatocytes cultured for 7 weeks after isolation using 12 hours of warm ischemia showed 1.2% of normal FVIII function. The Applicants believe without wishing to be bound by any theory that ischemia during isolation may result in a more robust and virile hepatocyte population in which hepatocyte secretory cell function is maintained in long-term culture.

Non-parenchymal cells (NPC) were isolated from the same liver used for hepatocyte isolation. Cell and yield was low, has been previously reported (Gerlach, 2001). Despite this, NPC so isolated were competent to produce FVIII, and indeed were producing approximately the same amount of FVIII as hepatocytes (approximately 0.2%) with about one-third the amount of cells.

Epithelial and endothelial cells from piglet gall bladder and liver vessels were isolated. These cells showed good growth in culture, proliferated under the culture conditions used, and exhibited the highest rate of FVIII coagulation, at 3.7%.

#### **EXAMPLE 6**

The effects of cell isolation methodology and culture conditions on cell viability and maintenance of secretory cell function for mouse hepatocytes were assessed. Additionally, methods to encapsulate mouse hepatocytes as single cells and as cell clusters were developed.

Six adult CD1 mice, one month of age, were used for isolation of adult mouse hepatocytes. Isolation of neonatal mouse hepatocytes was performed using

12 5-7 day old CD1 mice and repeated with 45 5-7 day old CD1 mice. The average weight of the isolated neonatal livers was approximately 0.07 grams.

Hepatocytes were isolated from mouse liver following the standard procedure described herein with three rounds of digestion for 10 min using Liberase® at a concentration of 0.2mg/ml. After isolation, cells were counted and plated in 25 cm<sup>2</sup> flasks.

Cells were isolated using the cold ischemia method as follows. Liver was cut into small pieces, and put in a large volume of cold DMEM and stored for 24 hours at 4°C. Hepatocytes were then isolated following the standard procedure described herein using Liberase® (0.2 mg/ml).

Viability of hepatocytes after isolation from adult mouse liver was 49%. Viability of adult mouse hepatocytes after isolation using the cold ischemia protocol was 2%. Cell count visibly dropped during the 1-2 weeks in culture. ICG showed positive staining for hepatocytes, see Figures 4, 5, and 6. Cells did not survive longer than 2 weeks in culture.

Viability of cells immediately after isolation from neonatal mouse liver was 31% and 23% for the first and second preparations, respectively. Cells were cultured in routine media supplemented with 10% FBS. After one week in culture hepatocytes were encapsulated as follows. Briefly, 1.5% alginate was used for the formation of capsules (Chang, 1994), according to the attached Protocol.

Figure 7 and 8 show single cells in capsules. Figure 9 shows a cell cluster in capsule. The integrity of capsules is shown in Figures 10, 11, and 12. No cells were visibly embedded in the capsule wall, and capsules were of even shape and size, approximately (200 µm in diameter).

### *Discussion*

The standard isolation technique with low Liberase® concentration (0.2 mg/ml) yielded lower cell viability with mouse hepatocytes than that observed with pig hepatocytes.

Although viability of neonatal hepatocytes after isolation was lower than that of adult hepatocytes (31% and 23% compared to 49%), neonatal cells showed much better survival during culture. Cell count will follow.

Mouse hepatocytes encapsulated in alginate (1.5%) resulted in capsules of good shape and integrity, with no cells embedded in the capsule walls, indicating that liver cells from mammals other than pigs are amenable to aggregate formation and encapsulation.

#### **EXAMPLE 7**

The effects of cell isolation methodology and culture conditions on cell proliferation and secretory function were assessed.

##### *7.1 Cell proliferation*

Neonatal hepatocytes were isolated according to our standard method described herein. 250,000 cells were seeded in flasks, and cultured in growth media supplemented with 10% FBS. Proliferation of cells in culture was determined at 1 day in culture, 7 days in culture, 14 days in culture, and 21 days in culture.

The number of viable cells, relative to day 1 of culture, in growth media supplemented with 10%FBS was 20% at day 7, 203% at day 14, and 287% at day 21.

##### *7.2 Albumin release*

Hepatocytes in growth media supplemented with 10% PS or 10% FBS were cultured, and albumin release was checked as described above at 1, 2, 3, 7, 8, and 9 weeks of culture. Pig fibroblasts in growth media supplemented with 10% PS or 10%FBS were used as a negative control in this experiment setting.

Maximum albumin release for hepatocytes cultured in growth media supplemented with 10% porcine serum was observed at week 9 (19.1  $\mu\text{g/ml}$  for 4 hours). A rise in albumin production was observed in the negative control, with a maximum albumin release of 0.67  $\mu\text{g/ml}$  at week 3, see Figure 13.

#### *Discussion*

The low cell viability at day 7 of culture indicated that the hepatocytes were damaged during the isolation step. However, under these conditions, the hepatocytes were able to recover and proliferate.

Albumin production was inhibited for several weeks in culture with albumin release considerably lower than in previous study. Again however, under these culture conditions, secretory cell function recovered after 8-9 weeks in culture.

Hepatocytes isolated and cultured as described can be successfully cultured to maintain hepatocyte-specific cell function, including secretory cell function, for at least 9 weeks *ex vivo*.

#### EXAMPLE 8

The effects of isolation methodology and culture conditions on secretory cell function of isolated pig hepatocytes were assessed as follows.

One week old AF SPF3 piglets were used for hepatocyte isolation.

Hepatocytes were isolated from one neonatal pig liver according to our standard procedure described herein, with two rounds of digestion with Liberase® (0.2mg/ml) for 10 min. After isolation, cells were counted and plated in 25 cm<sup>2</sup> flasks at  $4.5 \times 10^6$  cells/flask.

Hepatocytes were also isolated using the cold ischemia method described herein. Briefly, liver was cut into small pieces and put in a large volume of cold DMEM and stored for 24 hours at 4°C. Hepatocytes were isolated following the standard procedure described above.

Viability of the cells immediately after isolation using the standard procedure was 97%, and 86% after isolation using the cold ischemia method. Cells were seeded at  $2 \times 10^6$  cells per flask. Cell count dropped dramatically at day 2 in culture then increased to exceed the original level after two weeks in culture, see Figure 14.

Tests on albumin and Factor VIII release were performed on the supernatant of cultured cells as described above.

Hepatocytes isolated using the cold ischemia method showed higher albumin release than hepatocytes isolated according to the standard protocol at each time point, see Figure 15.

For both hepatocytes prepared according to the standard protocol and hepatocytes isolated using the cold ischemia method, maximum FVIII secretion was observed after 3 weeks in culture, see Figure 16. Maximal rate of FVIII coagulation was 0.12 units/ml/ $10^6$  cells (or 5.3% per  $1.6 \times 10^6$  cells).



Epithelial and endothelial cells were isolated from pig gall bladder and liver vessels as follows. Briefly, gall bladder was thoroughly washed with sterile DMEM to remove bile, cut in pieces, and digested for 30 min Liberase® solution (with 0.2 mg/ml). Cells were then washed with DMEM three times. Cell count and viability tests were performed immediately after isolation, and at day 7, day 16, and day 28 in culture. Cells were plated at  $15 \times 10^6$ /flask in  $25\text{cm}^2$  flasks.

Considerable FVIII release by epithelial and endothelial isolated from pig gall bladder and liver vessels as described above was observed after 4 weeks in culture, see Figure 16.

Gall bladder cell preparation showed considerable amount of Factor VIII release after 4 weeks in culture.

#### *Discussion*

Again, the Applicant's standard isolation technique using low Liberase® concentration provides good results with respect to cell yield and viability. In this instance, the viability of cells isolated using the cold ischemia protocol was comparable to that achieved using the standard method, of 86%.

Hepatocytes cultured following cold ischemia isolation showed better functional recovery compared to those isolated by the standard procedure, as demonstrated by albumin release.

Irrespective of the isolation method used, the cultured cells maintained secretory cell function after 4 weeks in culture, with maximum FVIII secretion observed at 3 weeks in culture for hepatocytes. Epithelial and endothelial cells isolated from pig gall bladder and liver vessels exhibited significant FVIII secretion after 4 weeks in culture, in excess of that observed at the same timepoint with hepatocytes.

All patents, publications, scientific articles, and other documents and materials referenced or mentioned herein are indicative of the levels of skill of those skilled in the art to which the invention pertains, and each such referenced document and material is hereby incorporated by reference to the same extent as if it had been incorporated by reference in its entirety individually or set forth herein in its entirety. Applicants reserve the right to physically incorporate into this



specification any and all materials and information from any such patents, publications, scientific articles, web sites, electronically available information, and other referenced materials or documents.

The specific methods and compositions described herein are representative of various embodiments or preferred embodiments and are exemplary only and not intended as limitations on the scope of the invention. Other objects, aspects, examples and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitation or limitations, which is not specifically disclosed herein as essential. Thus, for example, in each instance herein, in embodiments or examples of the present invention, any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms in the specification. Also, the terms "comprising", "including", "containing", *etc.* are to be read expansively and without limitation. The methods and processes illustratively described herein suitably may be practiced in differing orders of steps, and that they are not necessarily restricted to the orders of steps indicated herein or in the claims. It is also that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a host cell" includes a plurality (for example, a culture or population) of such host cells, and so forth. Under no circumstances may the patent be interpreted to be limited to the specific examples or embodiments or methods specifically disclosed herein. Under no circumstances may the patent be interpreted to be limited by any statement made by any Examiner or any other official or employee of the Patent and Trademark Office unless such statement is specifically and without qualification or reservation expressly adopted in a responsive writing by Applicants.

The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intent in the use of such terms and expressions to exclude any equivalent of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention as claimed. Thus, it will be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

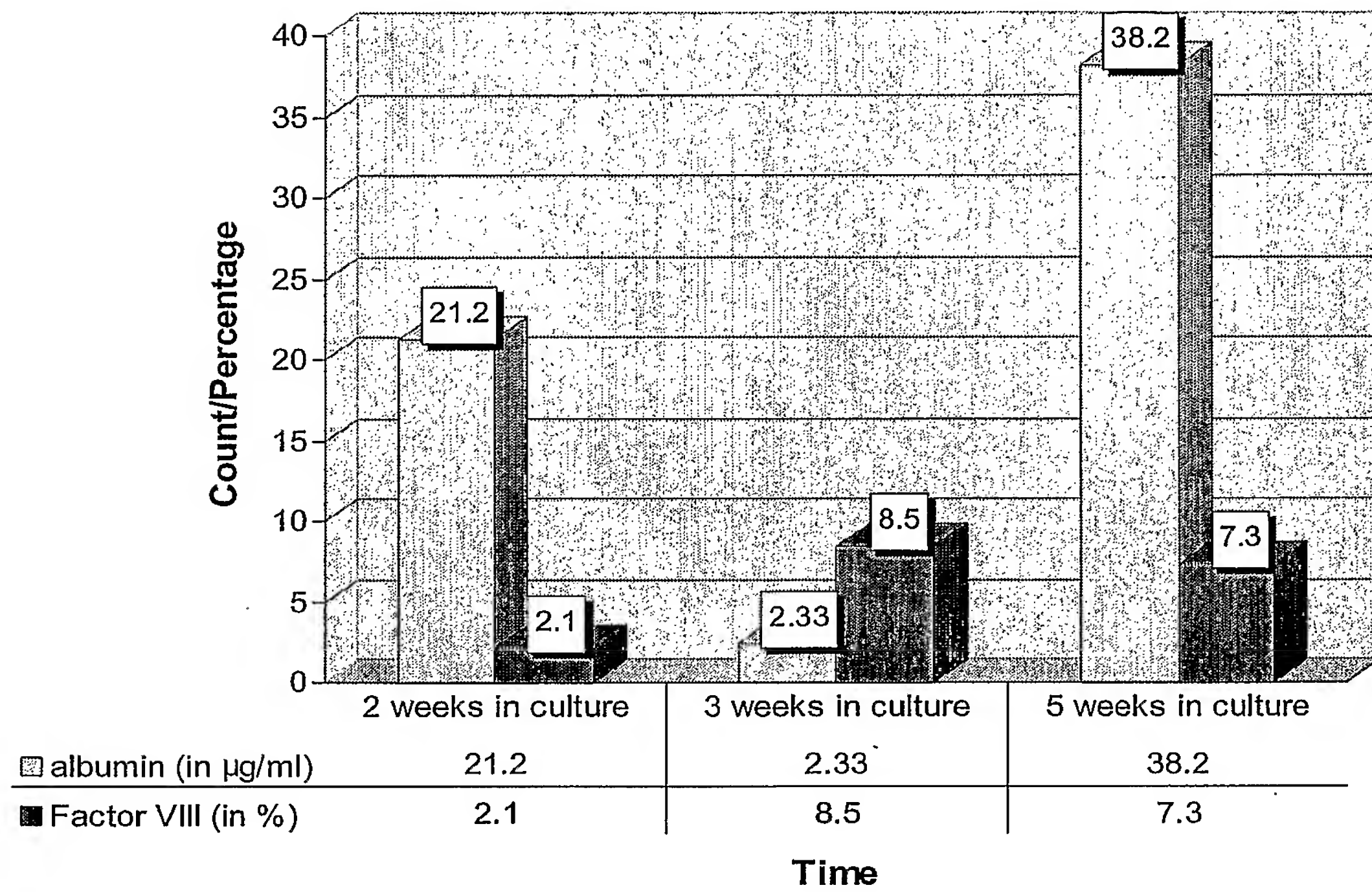
DATED THIS 30<sup>th</sup> DAY OF March 2004  
AJ PARK  
PER *Hensdale*  
AGENTS FOR THE APPLICANT

Intellectual Property  
Office of NZ

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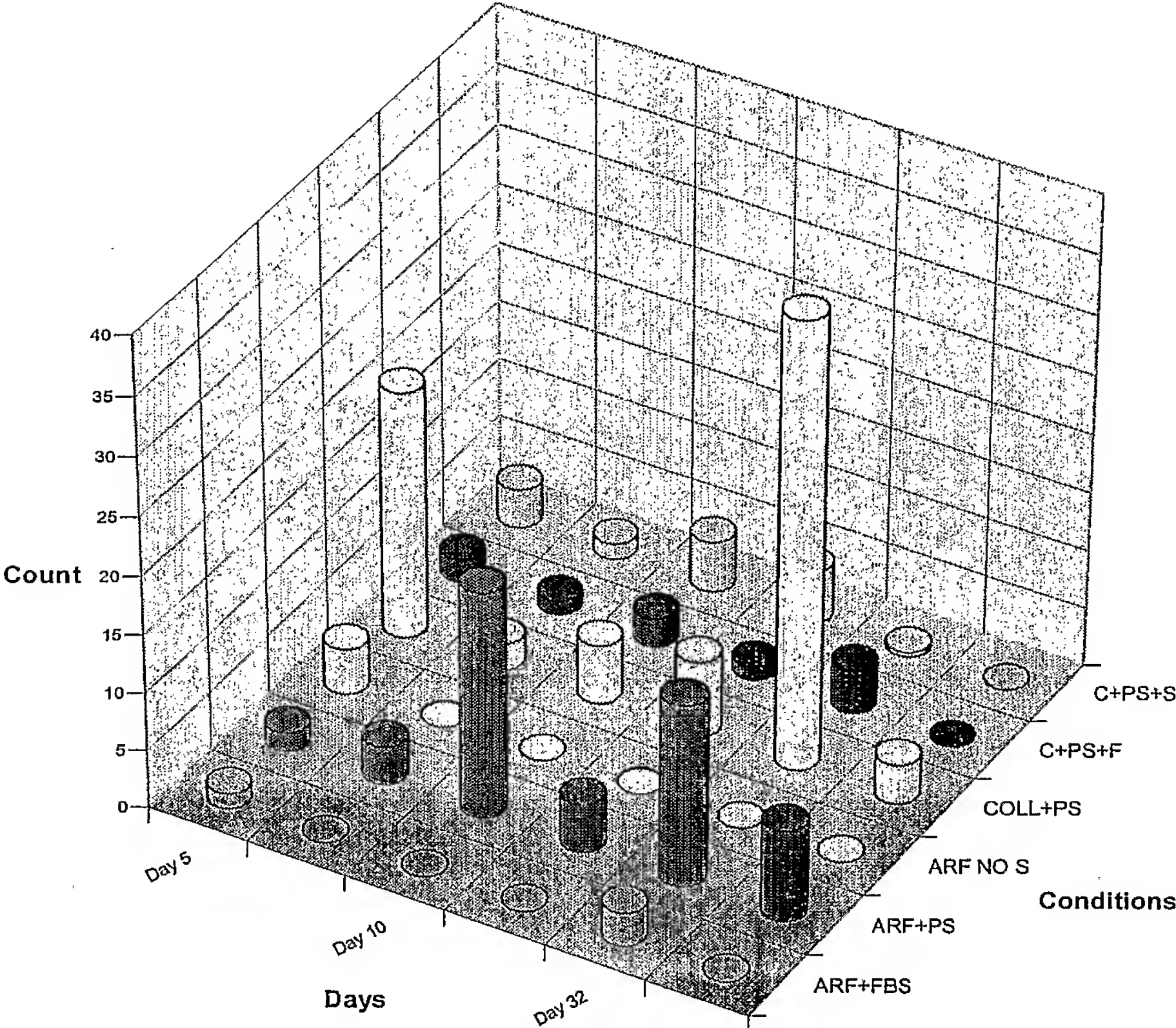
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# Albumin and Factor VIII production



**Figure 1**

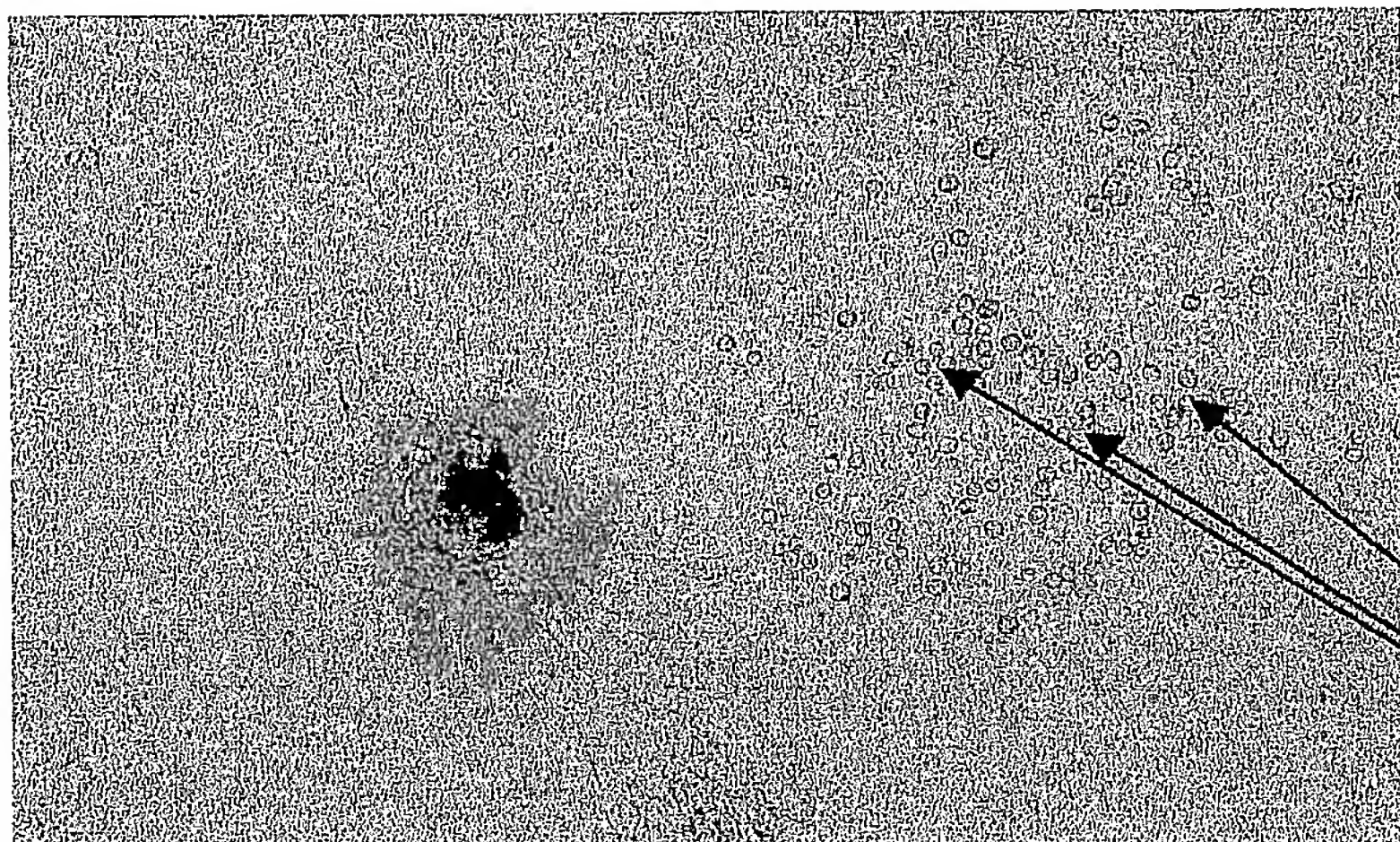
Albumin Production



	Day 5	Day 7	Day 10	Day 13	Day 32	Day 54
ARF+FBS	1.3	0	0	0	2.9	0
ARF+PS	1.6	3.3	19.5	4.2	15.7	7.9
ARF NO S	3.9	0	0	0	0	0
COLL+PS	21.2	2.33	5.06	6.5	38.2	3.42
C+PS+F	2	1.2	2.48	1.16	3.76	0
C+PS+S	3.3	1.34	4.34	4.56	0.36	0

Figure 2





ICG positive cells

Figure 3

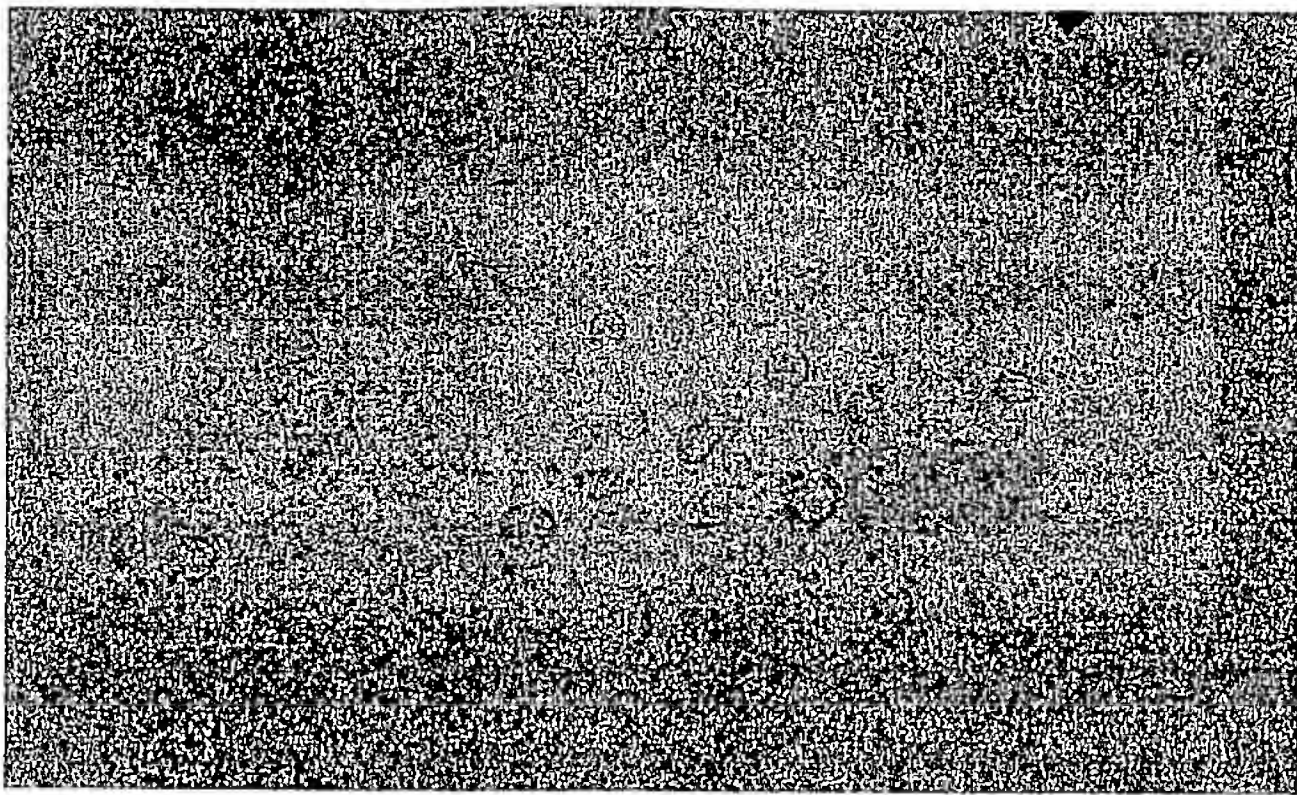


Figure 4

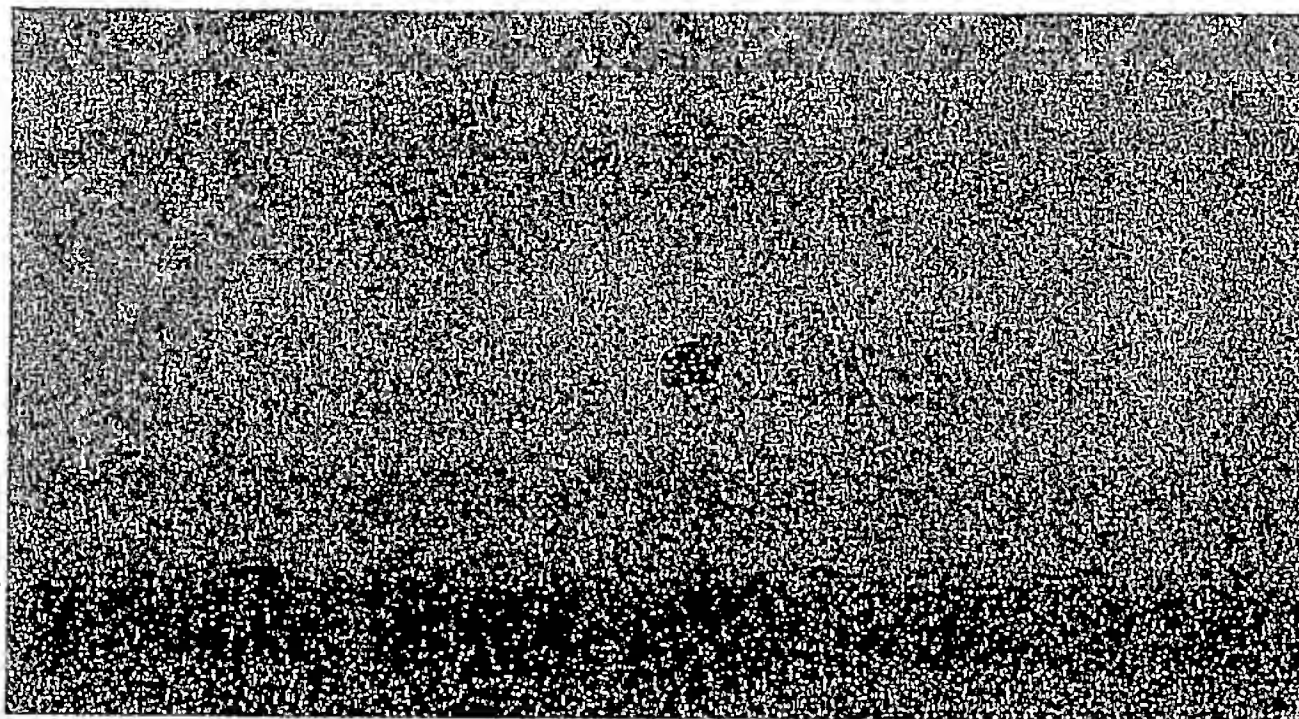


Figure 5

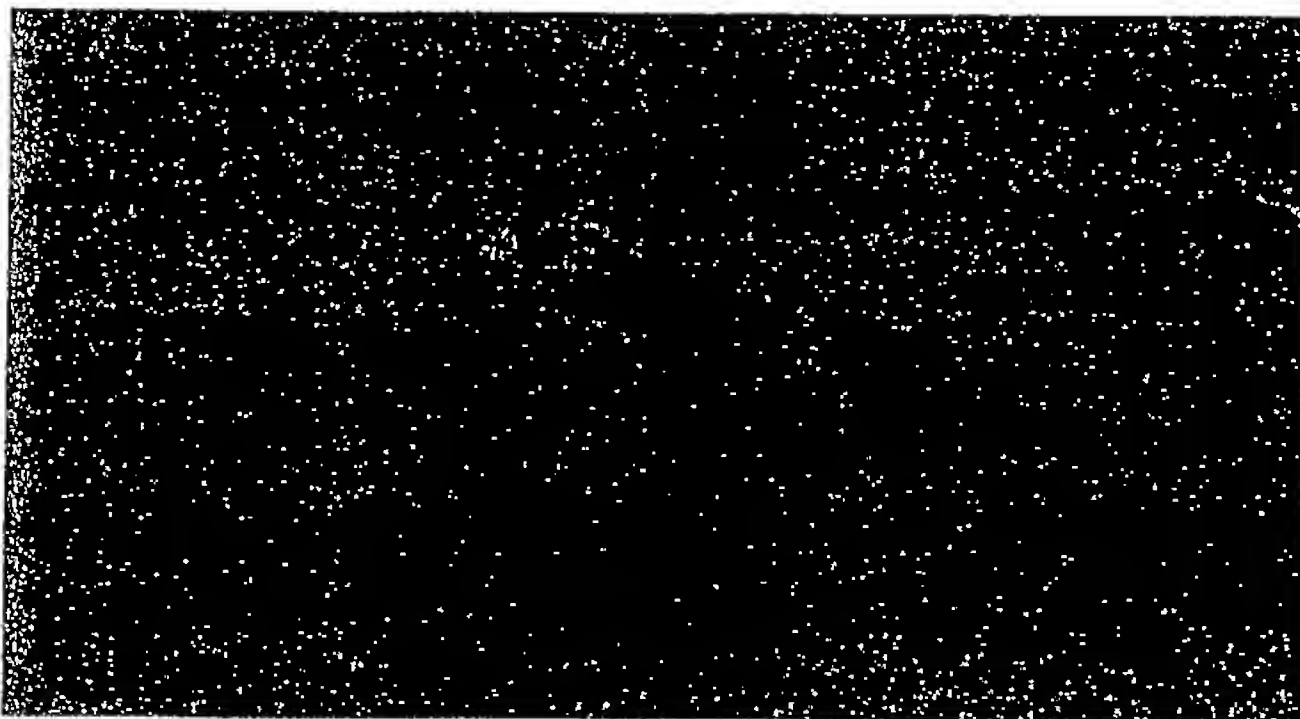


Figure 6



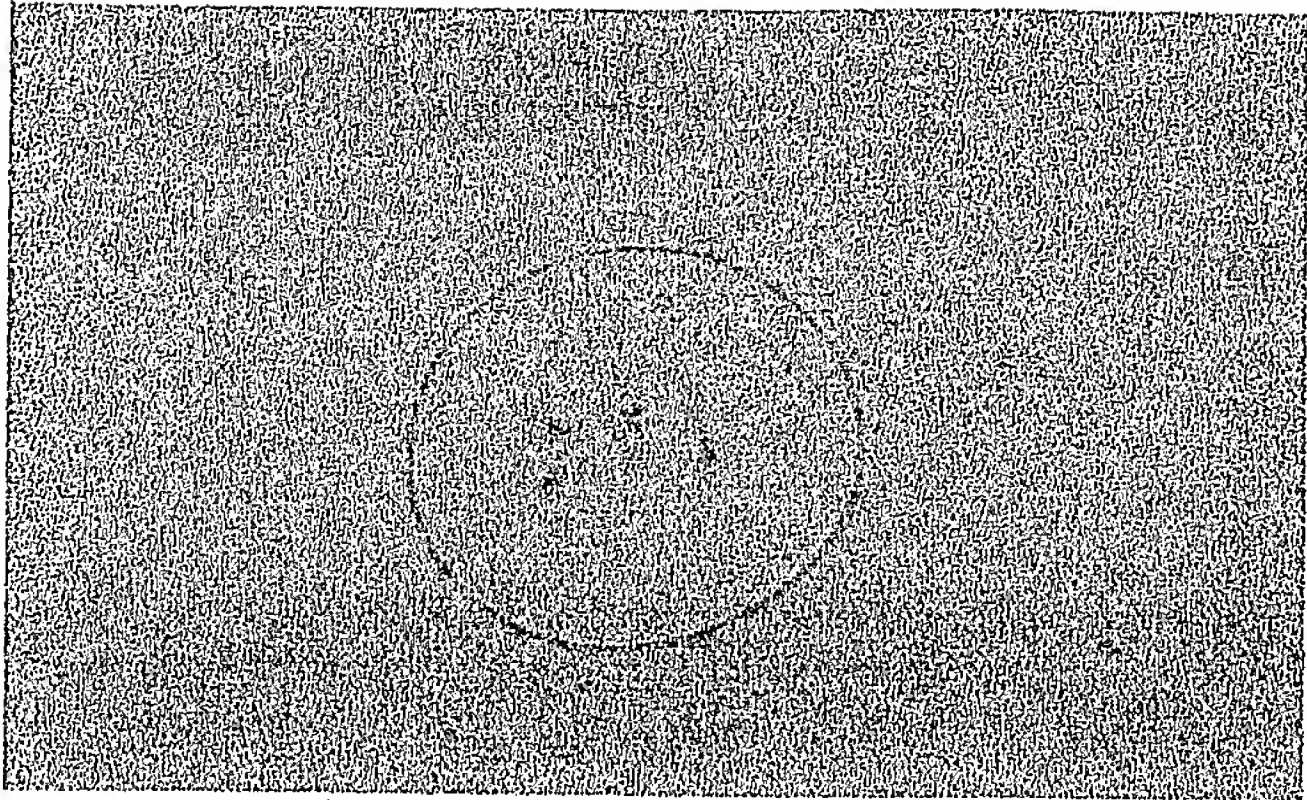


Figure 7

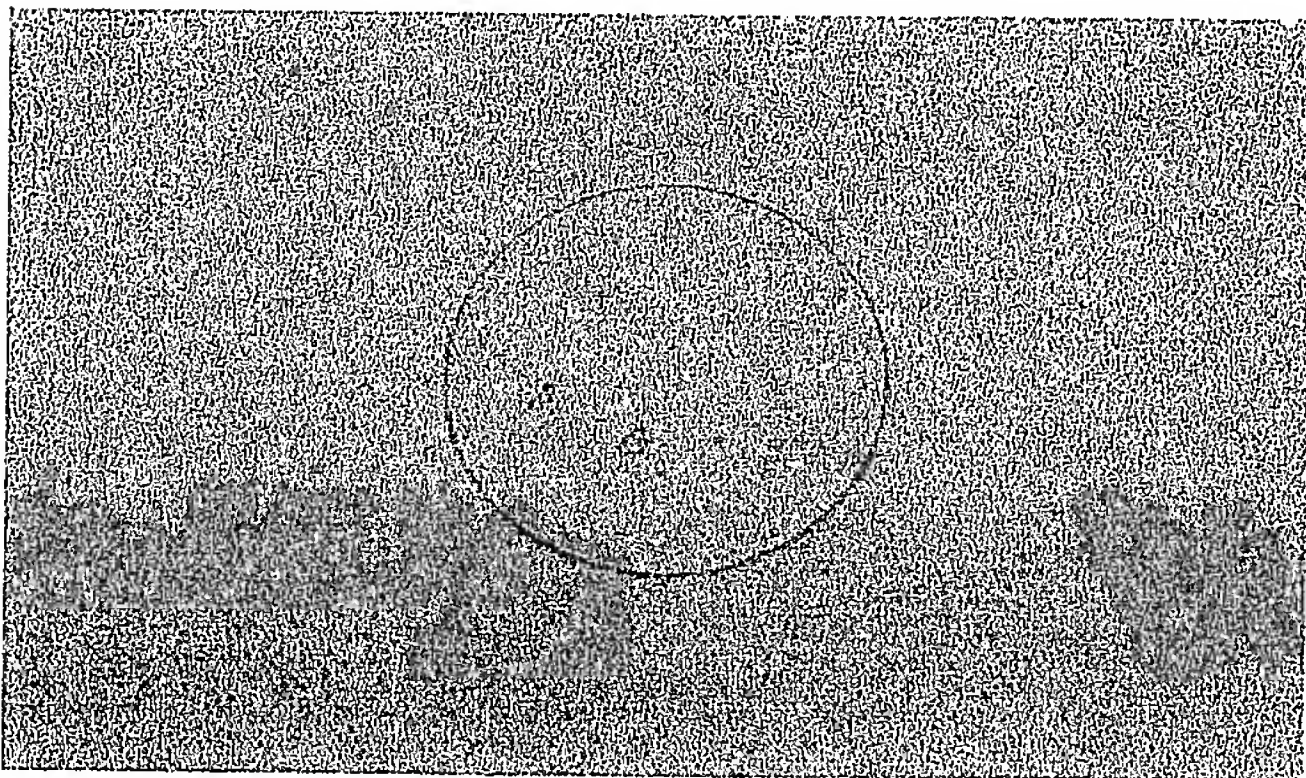


Figure 8

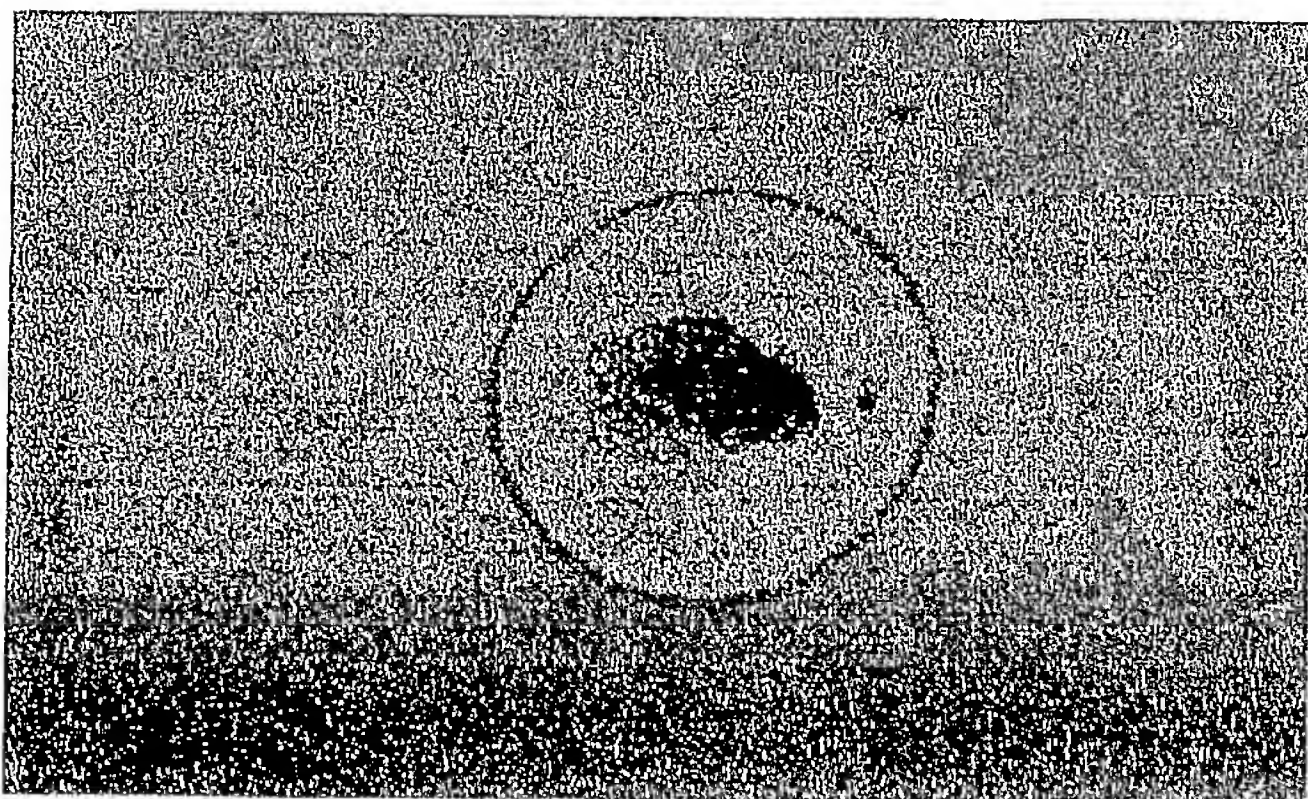


Figure 9

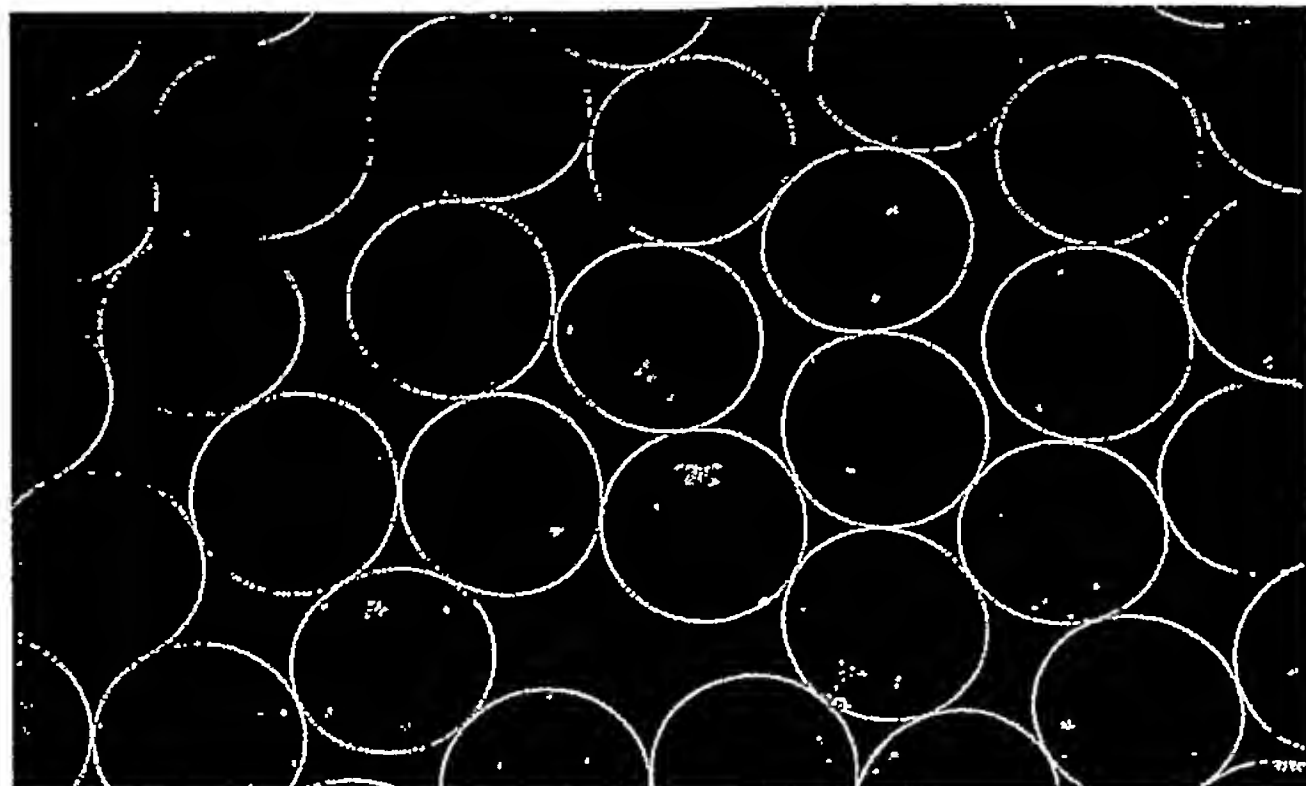


Figure 10



Figure 11

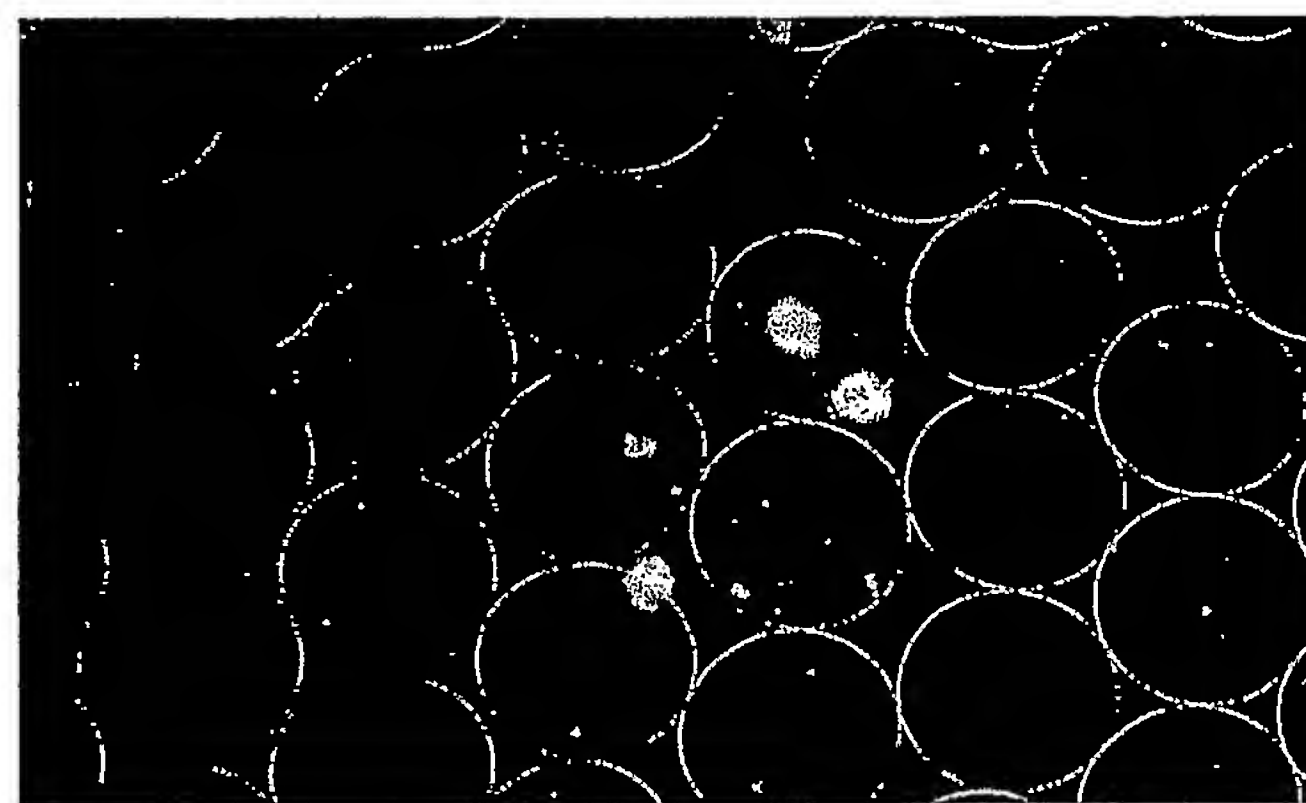
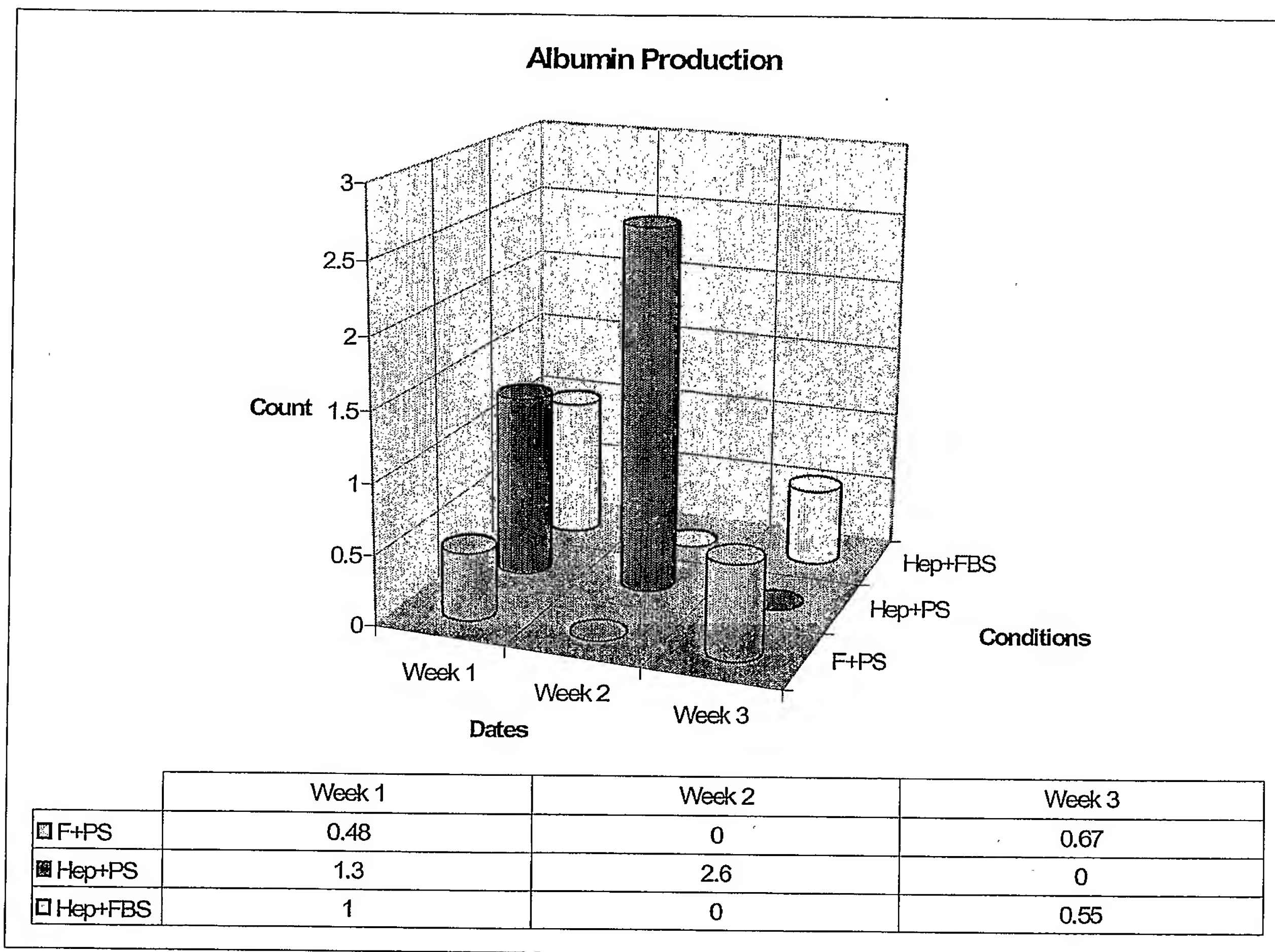
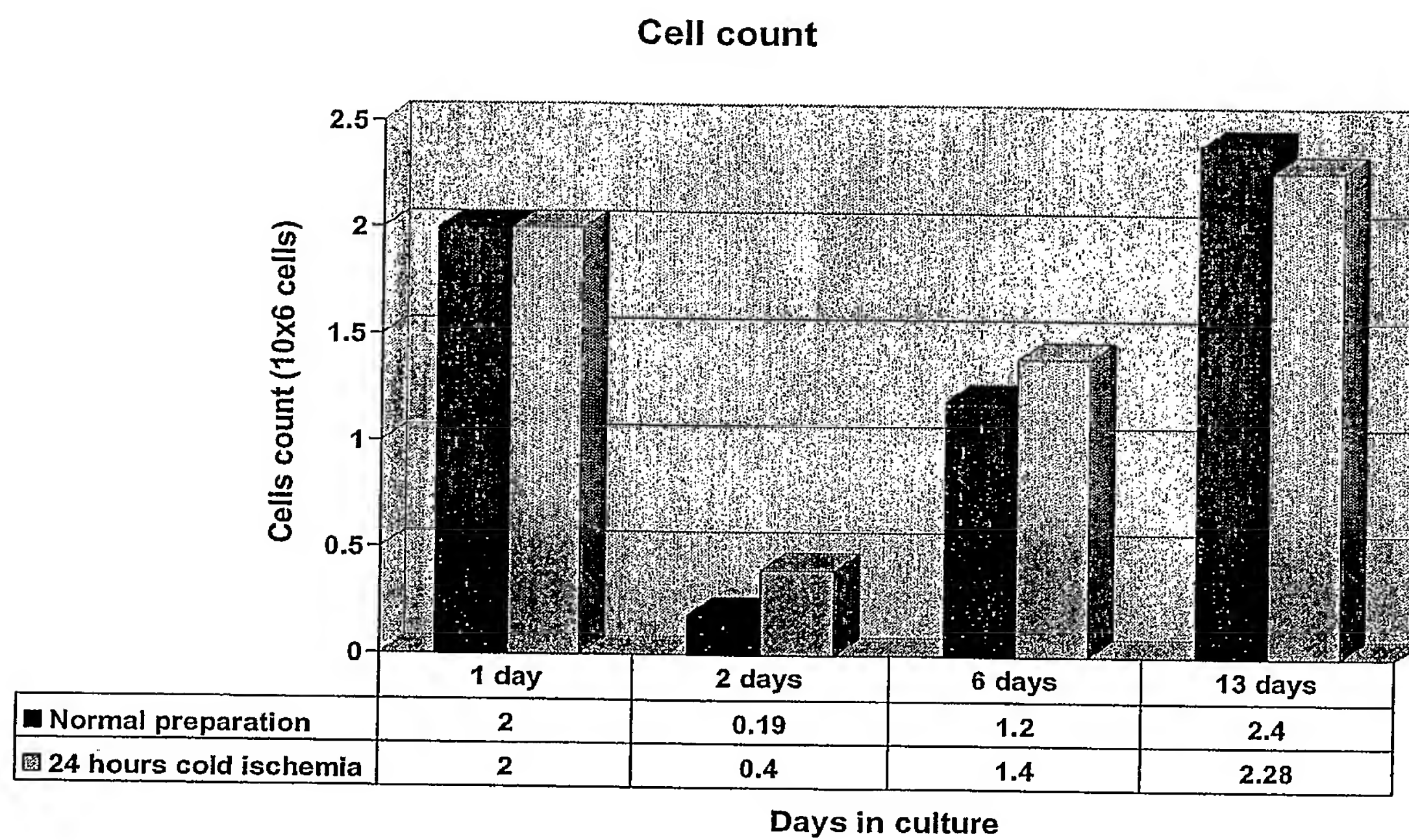


Figure 12





**Figure 13**



**Figure 14**

Albumin release

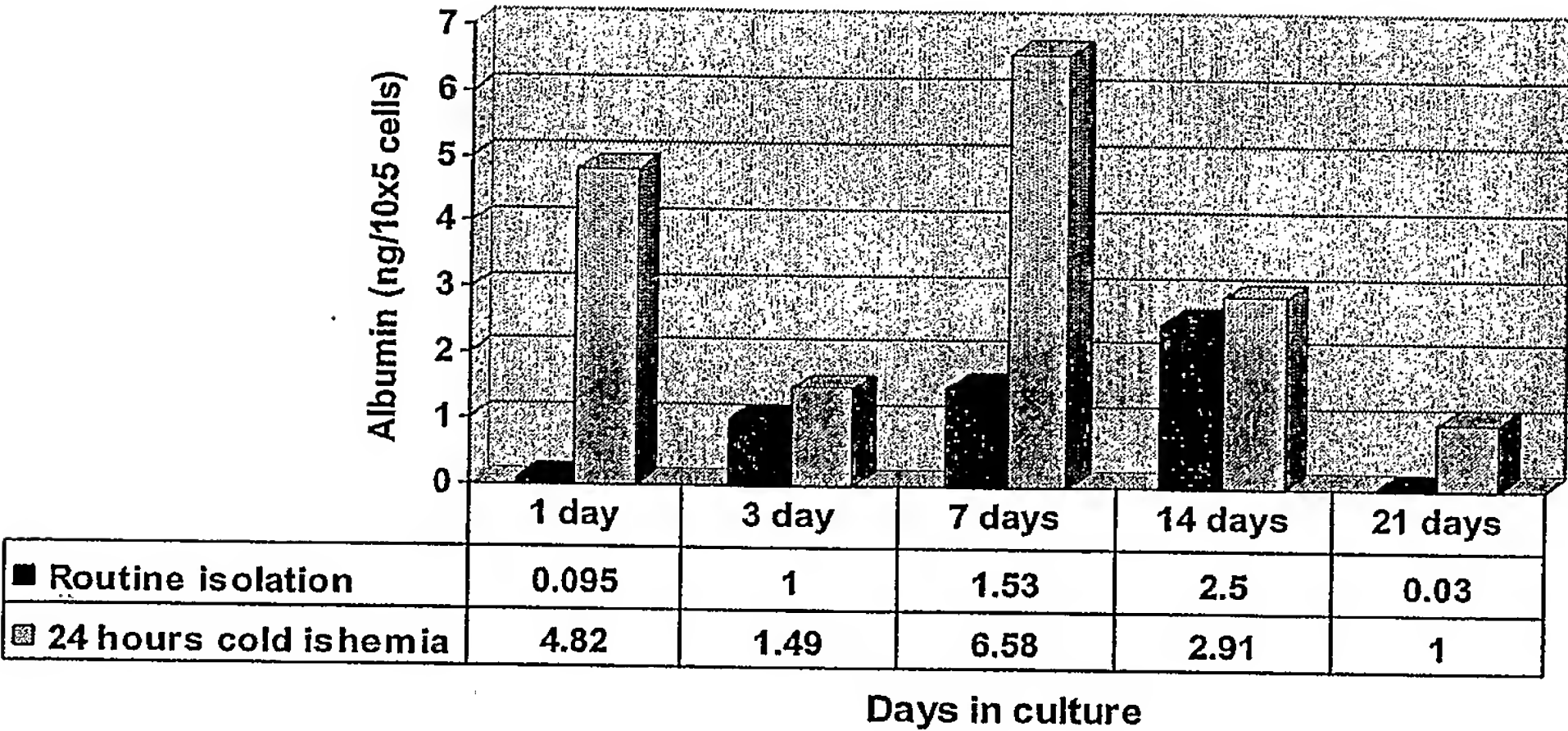


Figure 15

## Factor VIII release

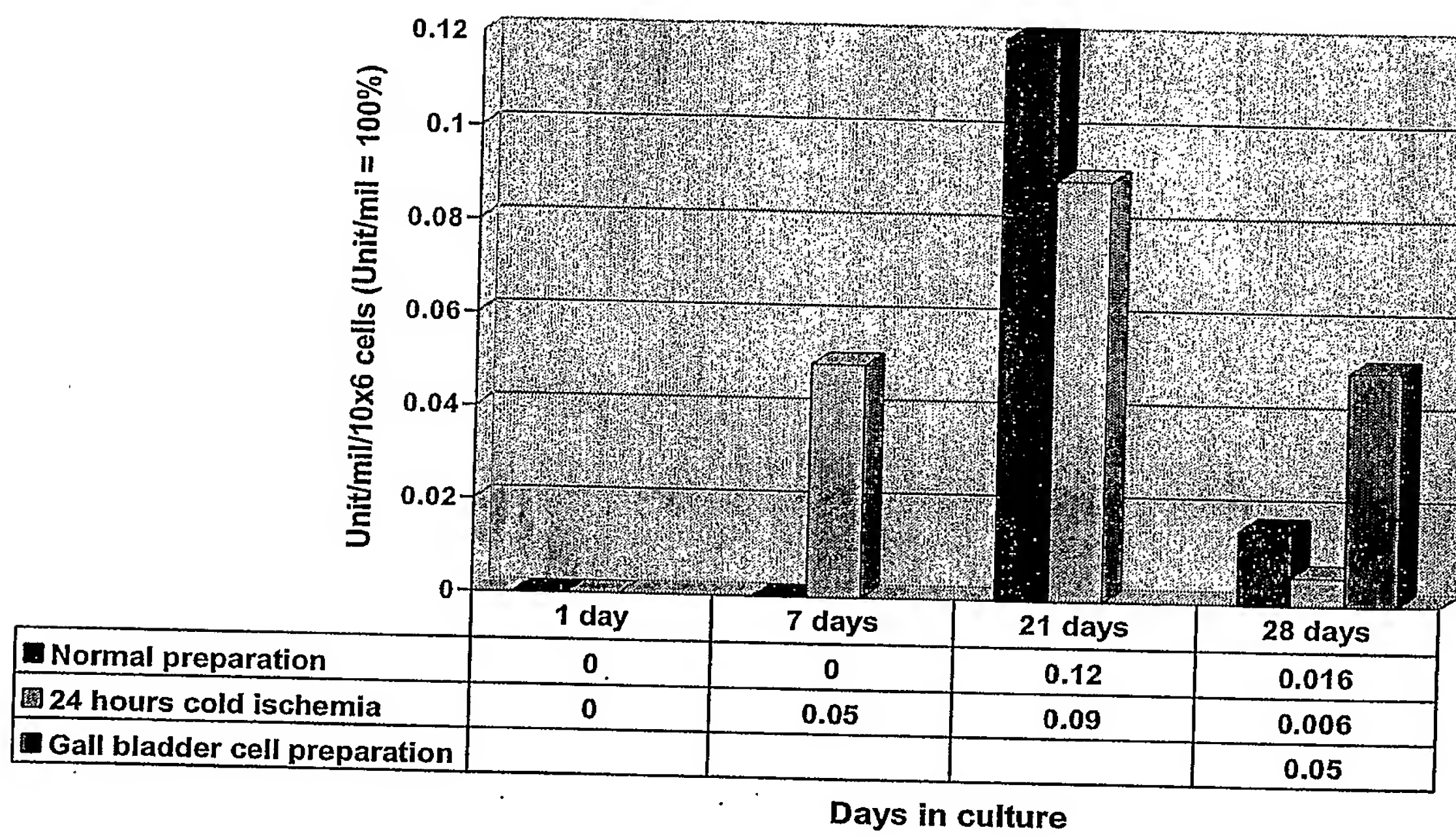


Figure 16